University of Alberta

Activity and Isoform Specific Distribution of AMP-Activated Protein Kinase in Rat Skeletal Muscle of Different Fibre-Types

by

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Abstract

The purpose of this study was to characterize the total activity and isoform-specific distribution of AMPK in slow, intermediate and fast-twitch rat muscle. Electrophoretic and immunohistochemical analyses revealed soleus (SOL), and white gastrocnemius (WG) were composed principally of slow MHC-I, and fast MHC-IIb and -IId/x, respectively, while red gastrocnemius (RG) contained primarily MHC -IIa. WG yielded the highest total AMPK activity and content. Type IIb fibres and Type I fibres predominately expressed alpha-2 and alpha-1 isoforms respectively, while Type IId(x) and Type IIa fibres expressed both isoforms. In SOL and WG, both alpha isoforms were localized to the nucleus. However, there was a 6-fold greater alpha-2 expression in the nuclei of the WG. These data support the hypothesis that fast twitch IIb fibres may require greater amounts of AMPK. Moreover, the alpha-2 isoform may have a more significant role in signalling transcription in the nuclei of fast rather slow-twitch fibres.

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Abbreviations

A priori	in advance
ADP	5'-adenosine diphosphate
AMP	5'-adenosine monophosphate
ATP	5'-adenosine triphosphate
BSA	bovine serum albumin
CEB	cellular energy balance
CoA	Coenzyme A
Cr	creatine
ddH_2O	double distilled water
DTT	dithiotreitol
ECL	enhanced chemiluminesence
EDTA	ethylenediaminetetraacetic acid
EGTA	Ethylenebis(oxyethylenenitrilo)tetraacetic acid
In vivo	within a whole, living organism
kDa	kilo-Daltons
PEG	polyethyleneglycol
PCr	phospho-creatine
SDS-PAGE	sodium dodecyl Sulfate Polyacrylamide Gel Electrophoresis
ZMP	AICAR 5'-monophosphate
ZTP	AICAR 5'-triphosphate

Nomenclature

1. Energy charge

Originally coined by Daniel Atkinson in the 1960s, energy charge likens a cell to a battery, in that it can be "charged" or "discharged" according to the concentrations of nucleotides. When referring to AMPK, energy charge is now calculated as the ATP-to-AMP ratio.

2. Cellular fuel gauge

A theory of AMPK function that proposes that AMPK is responsible for detecting the energetic state of the cell much like the fuel gauge in a car.

3. Plasticity

Relating to the intrinsic ability of muscle to alter its phenotype and therefore its functional properties in response to imposed environmental demands.

Chapter I

Introduction

Skeletal muscle is a multifaceted tissue that possesses remarkable adaptive capabilities. Each muscle contains a myriad of fibre types, whose structural and functional parameters span a theoretical continuum ranging from fast-twitch, glycolytic to slow-twitch, oxidative fibres. Where a fibre is situated on this continuum is dependant upon its specific contractile activity (Staron and Pette, 1986). Each muscle possesses a customized battery of characteristics that are uniquely suited to the specific demands placed upon it. The properties of these individual fibre types, in concert with the proportions of each fibre type ultimately determine the functional properties of the whole muscle. Any chronic alteration in these contractile demands signals a logical and sequential adaptation within each fibre, denoted by a phenotypic transformation tailored to match these new functional demands.

Historically, muscle fibres have been classified based on many diverse variables including structural and contractile properties, expression of myofibrillar protein isoforms, and metabolic enzyme profiles (reviewed by Pette & Staron, 2001). However, the presently accepted method for distinguishing fibre type is by analysis of their myosin heavy chain (MHC) content. MHC is the most important myofibrillar protein and is highly correlated with velocity of contraction in a fibre (Bottinelli et al., 1991) and

myofibrillar adenosine triphosphate (mATPase) activity (Staron & Pette, 1986). Although hybrid fibres do exist which express two neighbouring MHC isoforms, muscle fibre types are delineated according to the expression of the corresponding pure MHC isoforms. These are, in slow-to-fast order, type I, IIA, IID/X or IIB.

Repeated stimulation of skeletal muscle fibres, either through chronic low frequency stimulation (CLFS) or endurance training induces a fast-to-slow transformation (Pette and Staron, 2001). Increases in neuromuscular activity or mechanical loading conditions also result in a shift towards a more energy-efficient fibre type. This adaptation facilitates an enhancement of ATP production, and therefore an increased endurance during higher intensity activity. Despite the fact that the plastic nature of skeletal muscle has been well documented, the mechanisms involved in signalling this phenotypic transformation remain unclear. Several pathways have been targeted as possible signalling agents in this process, including a relatively recent addition to this list, 5'-adenosine monophosphate - activated protein kinase (AMPK)(Winder et al., 1996).

AMPK is a highly conserved heterotrimeric enzyme complex that acts as a metabolic "fuel gauge" in eukaryotic cells (Hardie et al., 1998). During conditions such as heat shock, nutrient starvation or vigorous exercise, AMPK is activated via a complex process that involves allosteric regulation, inhibited phosphatase activity, and phosphorylation by one or more upstream AMPK kinases (AMPK-K) (Hardie et al., 1998, Kemp et al., 1999). AMPK responds

to acute disturbances to the intracellular energy balance (IEB) by phosphorylating multiple metabolic targets which "switch off" anabolic pathways and "switch on" catabolic pathways, counteracting acute IEB perturbations (Winder, 2001).

The IEB is represented by the ratio of ATP-to-AMP. During acute contractile activity, this ratio decreases as ATP is hydrolysed to facilitate contraction. The extent to which the ratio is decreased varies between different fibre types (Conjard et al., 1998). When compared to slow-twitch fibres, those fibres situated nearer to the fast-twitch end of the continuum develop greater maximal isometric tension (Bottinelli et. al., 1994), exhibit greater unloaded contractile velocities (Galler et al., 1994), rely primarily on stored high-energy phosphates and glycogen as substrates and are recruited only during high-intensity contractile activity. As such, these fast-twitch fibres experience greater disturbances to the cellular energy balance than slower-twitch fibres (Conjard et al., 1998), and are referred to as having "loose" metabolic control. Conversely, slow-twitch fibres exhibit a much "tighter" control over the energy balance, quickly restoring cellular energetics to a state of equilibrium following acute perturbations.

In addition to the role it plays in regulating cellular energetics, AMPK may also have a secondary function as a central downstream component of a second messenger cascade involved in regulating skeletal muscle gene expression. It is conceivable that chronic activation of AMPK through activity-induced stimulation will lead to the transformation of strictly glycolytic, faster-

twitch fibres toward a more oxidative, slower-twitch phenotype. Features central to mechanisms regulating gene expression in any tissues are the selective expression or covalent modification of transcription factors, which interact with the upstream regulatory regions associated with the transcribed region of the gene.

The $\alpha 2$ containing AMPK isoform may be ideally suited to regulate muscle fibre phenotype for several reasons; its primary biochemical function is phosphorylation, it has been localized in the nuclei of hepatocytes (Salt et al., 1998) and skeletal muscle (Ai et al., 2003), and it has been found to regulate transcription of glucose responsive enzymes. These enzymes include glucose-6-phosphate in liver and phosphoenolpyruvate carboxykinase in skeletal muscle (da Silva Xavier et al., 2000; Woods et al., 2000; Winder et al., 2000; Zheng et al., 2001).

Changes in environmental stimuli that persistently depress the IEB within a muscle result in a fast-to-slow phenotypic transformation. While the mechanism responsible for signalling this response is unclear, a chronic change in cellular energetics is a logical stimulus. The ideal intermediary to transduce this metabolic signal is AMP-activated protein kinase, which responds to minute IEB perturbations. Additionally, AMPK has recently been shown to affect gene expression via transcriptional modification in skeletal muscle, making it a very probable candidate for regulating muscle fibre phenotype. The characterization of AMPK activity and distribution within the

diverse population of fibre-types within skeletal muscle is fundamental to a comprehensive understanding of AMPK and the cellular roles this protein kinase plays.

I.I Purpose of the Study

The purpose of the current study was to characterize the total activity and isoform-specific distribution of AMPK in different fibre-types of skeletal muscle, including its subcellular localization. The majority of AMPK studies on skeletal muscle have focused solely on the metabolic role of this enzyme in cellular fuel selection. The few studies that have attempted to characterize AMPK activity and content in mammalian skeletal muscle have done so without accurately taking into account the heterogeneity of fibre types that comprise skeletal muscle (i.e., type-I, IIA, IID/X and IIB fibre types) or differences in the distribution of the two known isoforms of AMPK between these fibre types. One very recent study (Ai et al., 2003) attempted to analyze the fibre-type specific distribution of AMPK via ATPase staining. However, they might have been better served delineating fibre types based on the more accepted classification of MHC expression.

To our knowledge, the proposed study will be the first to characterize the activity and distribution of the two known isoforms of AMPK, (i.e., $\alpha 1$ and $\alpha 2$), within the various fibre-type populations based on myosin heavy chain classification. These data will provide important insights into the cellular roles of these two isoforms within rodent skeletal muscle.

I.II Hypothesis

AMPK is a highly conserved and ultra sensitive signalling system activated to respond to metabolic stress. During prolonged bouts of contractile activity in skeletal muscle, cellular energetics are chronically strained. The extent to which the intracellular energy balance is perturbed occurs in a fibre-type specific manner (Conjard et. al., 1998). These differences in metabolic control between fibre-types, coupled with the role AMPK plays as an acute "metabolic manager", provide the foundation from which the hypothesis is built; skeletal muscle fibres with looser metabolic control would require a greater restorative intervention by AMPK to return to a homeostatic IEB. Therefore it is our primary hypothesis that fast-twitch fibres will have greater content and activity of AMPK than slow-twitch fibres.

On a more expansive note, the fundamental regulatory characteristics of AMPK might form the basis for long-term cellular adaptations commonly associated with exercise training. Such changes would facilitate long-term energy conservation by ameliorating the magnitude of subsequent perturbations to the IEB. This may be achieved by increasing the expression of slow cellular ATPase isoforms over their fast counterparts, thereby reducing the overall rate of ATP consumption. At the same time, the expression of oxidative enzymes is increased leading to an increase in the capacity for ATP production through complete substrate oxidation. Consequently, these coordinated adaptations would reduce the magnitude of subsequent energy deficits in skeletal muscle fibres during contractile activity. Indeed, recent

studies have demonstrated that chronic activation of AMPK by AICAR results in the increased expression of proteins which catalyze the uptake and phosphorylation of glucose (i.e., GLUT4 and hexokinase), as well as enzymes associated with complete glucose oxidation (Buhl et al., 2001; Winder et al., 2000; Holmes et al., 1999). Furthermore, because the expression of oxidative enzymes appears to be co-regulated with the expression of slow myosin heavy chain (MHC) ATPase isoforms (Dunn & Michel, 1997), it is possible that chronic activation of AMPK also leads to increased expression of slow and repression of fast MHC isoforms. Following this logic, it would be logical that as fibres shift towards the oxidative side of the continuum, AMPK expression and activity would be decreased. Interestingly, AMPK has been mentioned in a recent review as a potential candidate for regulating MHC expression during exercise training (Pette & Staron, 2000).

Thus it can be hypothesized that chronic activation of AMPK might lead to adaptations to facilitate long-term energy conservation strategies. Sustained activation of skeletal muscle AMPK could therefore induce compensatory changes in gene expression favouring an increased oxidative potential. This adaptation would involve the phenotypic transformation towards the more energy efficient, slow-twitch fibre. Therefore, a secondary hypothesis is that total and alpha-2-AMPK content will be highest in muscle composed primarily of fast-twitch fibres.

I.III Research Plan

In the first series of experiments, the fibre-type distribution of the two known isoforms of AMPK were examined by indirect immunohistochemistry on serial frozen sections followed by light microscopic analysis. Polyclonal antibodies that specifically recognise the $\alpha 1$ and $\alpha 2$ catalytic subunits were used to localize the two isoforms. Monoclonal antibodies that recognise the adult isoforms of MHC will be used to differentiate between slow (I), intermediate (IIA and IID/X) and fast (IIB) twitch fibres.

In the second experimental series, the content of the AMPK isoforms expressing the $\alpha 1$ and $\alpha 2$ catalytic subunits was quantified by Western Blot analysis and the maximal activity of AMPK was determined in rat skeletal muscles composed of different fibre types. The pharmaceutical agent aminodazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) was used to pharmacologically activate AMPK. Using a standard radiometric assay, total AMPK activity was examined by measuring the rate of incorporation of the terminal phosphate γ-^{32} P-ATP into a synthetic peptide (i.e., AMARA peptide), which has a phosphorylation motif specific to AMPK. After samples were re-suspended and all kinase reactions were complete, aliquots were removed and spotted on phosphocellulose paper to quantify radioactivity (β-emissions) by liquid scintillation counting.

I.V Limitations of the Proposed Study

The animals used in the present study were matched for species, age, gender and size. However, the fibre-type profiles of the selected muscles could not be independently controlled. Therefore muscles were selected that were previously shown to be predominately composed of fast-, intermediate- or slow-twitch fibres.

The efficacy of AICAR as a pharmaceutical activator of AMPK also limited the study. AICAR has been shown to activate AMPK in both rat and human muscle, however its mechanism for activation is through a simulated decrease in the IEB (ATP-to-ZMP rather than ATP-to-AMP), and its actions may not be specific to AMPK. Additionally, it may be a more potent stimulus for AMPK activity in fast-twitch than slow-twitch muscles, owing to the possible differences in their respective capacities for metabolism of ZMP (Sabina et al., 1982). Recognizing this possibility, CO_2 asphyxiation was used to euthanize the animals, placing them in a state of terminal anoxia prior to muscle extraction. Therefore, we coupled the AICAR treatment with another potent activator of AMPK, which should have provided a sufficient stimulus for maximal AMPK activity.

Additionally, the effectiveness of the synthetic AMARA peptide, and the specificity of the antibodies used in all of the immunohistochemical and immunoblot reactions may have posed possible limitations to the study. The efficacy of the AMARA would affect its ability to act as a substrate for phosphorylation by AMPK, while the specificity of the antibodies would affect

our ability to reliably differentiate between the two isoforms of AMPK. However, the anti-AMPK antibodies used in the study were commercially produced, and were previously shown to be specific to the respective isoforms they were engineered to identify. The AMARA peptide was produced at the Alberta Peptide Institute at the University of Alberta, in accordance with amino acid sequence reported (AMARAASAAALARRR) which contains the Hyd-X-Basic-X-X-Ser-X-X-X-Hyd motif (Salt et al., 1998).

The final limitation to the proposed study was the inability of the radiometric assay being utilized to completely measure total AMPK activity. The assay measures the largest component of AMPK activity, phosphorylation, by quantifying the incorporation of radiolabelled ATP into the AMARA peptide. However, the lesser component, the allosteric activation of AMPK by AMP cannot be measured by this procedure. Despite this, the radiometric assay that was used in the present study is the only known method of measuring AMPK activity at this time. Despite this potential limitation, the relative activity between muscles composed of different fibre types was distinguishable using this method.

Chapter II

Review of Literature

II.I A Continuum of Skeletal Muscle Fibre-Types

Skeletal muscle is a heterogeneous tissue, composed of muscle fibres that exist along a theoretical continuum (Figure II-1). At one end of the spectrum are primarily oxidative, slow-twitch fibres, and at the opposite end, glycolytic, fast-twitch fibres. Skeletal muscle is referred to as being "plastic" in nature, that is, each fibre-type possesses the ability to respond to chronic changes in stimuli by altering specific characteristics that allow it to meet the new contractile demands placed on it. The "plasticity" of skeletal muscle facilitates the adaptation of both metabolic and structural characteristics observed in response to chronic changes in contractile activity. Each specific fibre can be classified in several different ways, with the most accepted method being based on the myosin heavy chain content.

Myosin is a contractile protein composed of two heavy chains and four light chains. As many as eight myosin heavy chain (MHC) isoforms have been discovered, but four types appear to be predominately expressed in adult skeletal muscle (slow MHC-Iβ, and fast types MHC-IIa, -IId/x and –IIb) (Reviewed by Pette & Staron, 2002). The contractile phenotype of each muscle fibre is thus based on the expression of these MHC isoforms and fibres are correspondingly delineated as type I, IIA, IID/X and IIB. Myosin is

the ideal candidate to classify muscle fibre-types because of the important role it plays in determining the functional characteristics of the individual fibre, such as myofibrillar ATPase activity, velocity of contraction, and maximal isometric tension (MIT).

When compared to slow-twitch fibres, those fibres situated nearer to the fast-twitch end of the continuum develop greater MIT (Bottinelli et al., 1994), exhibit greater unloaded shortening velocities (Galler et al., 1994), rely primarily on stored high-energy phosphates and glycogen as substrates and are recruited only during high-intensity contractile activity. As such, these fast-twitch fibres experience greater disturbances to their cellular energy balance than slower-twitch fibres (Conjard et al., 1998), and as such are referred to as having "loose" metabolic control. Conversely, slow-twitch fibres exhibit a much "tighter" control over energy balance, quickly restoring cellular energetics to a state of equilibrium following acute perturbations.

However, the further towards the fast end of the continuum a fibre is situated, the greater it's capacity for a fast-to-slow phenotypic transformation. This is because the shift in both structural and metabolic characteristics occurs in an ordered manner. Peuker et al., (1998), showed that rabbit muscle fibres migrate sequentially across the continuum in response to chronic low frequency stimulation (CLFS). The continuum also represents a spectrum of energy efficiency, in that slow-twitch fibres generate more contractile tension per mole of ATP consumed compared to fast-twitch fibres (Bottinelli et al., 1994). When subjected to chronic stimulation such as endurance exercise

training, fibres shift towards a more energy efficient phenotype. This fast-to-slow transformation appears to be a compensatory adaptation, enabling the fibre to ameliorate future energy disturbances.

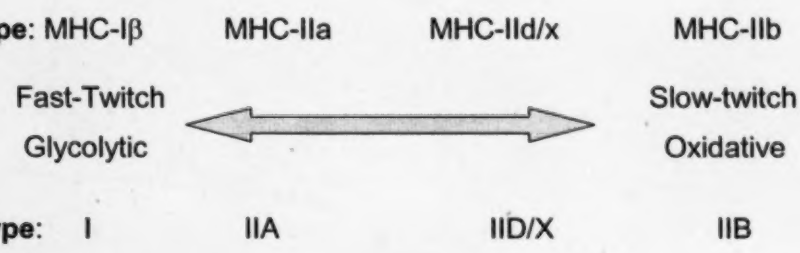

Figure II-1: Theoretical continuum of muscle fibre types

II.II Early History of AMPK

In 1973, Beg and colleagues first reported the activity of the protein kinase. Studying tissue from rat liver, they observed an inactivation of 3-hydroxy-3-methylglutaryl-CoA (HMG CoA) reductase, a major enzyme in cholesterol synthesis, when ATP was added to their samples. This inactivating factor was later realized to be a protein kinase and was named for its action, HMG CoA reductase kinase. Further research showed that AMP was a more potent stimulator of the kinase (Ferrer et al., 1985). Similar results were found when a protein kinase was also found to inhibit the actions of the enzyme responsible for controlling fatty acid synthesis, acetyl-CoA carboxylase (ACC) in rat liver cells (Hardie and Carling, 1986). This ACC-specific kinase also appeared to be highly activated by AMP.

Eventually it was discovered that HMG CoA reductase and ACC were both substrates of the same entity. This "new" protein kinase was named for its allosteric activator, AMP, and the name AMP-activated protein kinase was first used in publication in 1988 (Munday et al., 1988; Sim & Hardie, 1988). Since then, research investigating this multi-substrate kinase and its actions has flourished.

II.III Cellular Energy Balance

Maintenance of intracellular energy supply is a fundamental process for all healthy cells, and as such, complex mechanisms have evolved to regulate the ratio of ATP-to-ADP and related changes in the ATP-to-AMP ratio through the equilibrium enzyme adenylate kinase. Daniel Atkinson was the first to propose that these nucleotides themselves would be the signals that governed this type of regulation. In 1964, he and his colleagues theorized that the intracellular adenine nucleotides would mediate all catabolic and anabolic pathways calling this the *adenylate control hypothesis* or the *energy charge hypothesis* (Ramaiah et al., 1964). The authors likened the adenine nucleotide levels in a cell to the chemicals in a battery. ATP levels are increased via the actions of catabolic pathways, "charging the cellular battery", while anabolic processes deplete ATP stores, "discharging the battery".

All energy-requiring pathways within the cell are fuelled either directly or indirectly via the hydrolysis of ATP. The breakdown of ATP is catalyzed by the

action of two classes of enzymes: ATPases, yielding ADP and inorganic phosphate (Pi), and/or ATP ligases yielding AMP and pyrophosphate (PPi) (Figure II-2). The reactants and products of these two reactions are maintained many orders of magnitude away from their equilibrium ratios and during homeostasis, cells maintain an ATP-to-ADP ratio in the order of 10:1(reviewed by Hardie and Hawley, 2001).

ATPases: $ATP \rightarrow ADP + Pi$

Ligases: $ATP \rightarrow AMP + PPi$

ATP synthases: $ADP + Pi \rightarrow ATP$

Adenylate kinase: $2ADP \leftrightarrow ATP + AMP$

If adenylate kinase reaction is at equilibrium:

$$\frac{[ATP][AMP]}{[ADP]^2} = K$$

$$\therefore [ATP][AMP] = K \times [ADP]^2$$

Then divide both sides of the equation by $[ATP]^2$:

$$\therefore \frac{[AMP]}{[ATP]} \left(\frac{[ADP]}{[ATP]} \right)^2 \propto$$

$\therefore$ The AMP: ATP ratio varies as the square of the ADP: ATP ratio

Figure II-2: Reactions interconverting ATP, ADP, and AMP (Adapted from Hardie & Hawley, 2001)

Conversely, ATP is produced by the action of another class of enzyme, ATP synthase, which utilizes Pi to re-phosphorylate ADP. During energetic homeostasis, the action of the ATP synthases counters that of ATPases, maintaining the ATP-to-ADP ratio at 10:1. Under such conditions, net flux through the adenylate kinase reaction (ADP + ADP ↔ ATP + AMP) will flow from the right to the left, thereby maintaining an ATP-to-AMP ratio of 100:1 (Hardie & Hawley, 2001). However, when either the production of ATP is attenuated (e.g. during ischemia) or the rate of ATP consumption is elevated, (e.g. during exercise) the rate of ATPase activity will exceed that of ATP synthase. This will result in a depressed ATP-to-ADP ratio, a shift in the direction of flux through the adenylate kinase reaction, a concomitant increase in AMP production, and ultimately a decrease in the ATP-to-AMP ratio. Thus, as the ATP-to-AMP ratio varies as the square of the ATP-to-ADP ratio, the first ratio is an ideal candidate for representing cellular energy balance.

II.IV Regulation of AMPK

As the cellular energy balance of the cell is decreased, and the ratio of ATP-to-AMP decreases, AMPK is transformed through four different conformational states (Figure II-3). The first step in the process is the conversion of the less active form, AMPK-T, to a more active form AMPK-R. This results in a five-fold increase in activity and a subsequent increased affinity for upstream protein kinases, AMPK-K. Phosphorylation at the catalytic site, Thr^{172}, on the α subunit occurs via AMP-stimulated allosteric

activation of AMPK-K, increases activity further. Converting AMPK-R to its phosphorylated form, AMPK-R-P, results in a further ten-fold increase in enzyme activity. The complex mechanism of regulation described above allows minute increases in AMP concentration to be amplified, leading to complete conversion of AMPK to its most active state, AMPK-R-P.

Because ATP binding to AMPK is competitive with AMP, as ATP stores are replenished, AMP will become increasingly dissociated from both AMPK-R-P and AMPK-K. This will have the effect of decreasing AMPK-K activity and converting AMPK-R-P back to its less active form, AMPK-T-P. AMPK-T-P has a high affinity for protein phosphatase-2A (PP2A), which then dephosphorylates AMPK-T-P back to its inactivated state, AMPK-T. By sensing minute perturbations in the ATP-to-AMP and CP-to-Cr ratios of the cell, and functioning to restore the energy balance of the cell, AMPK seems to serve as a metabolic master switch regulating cellular energy state.

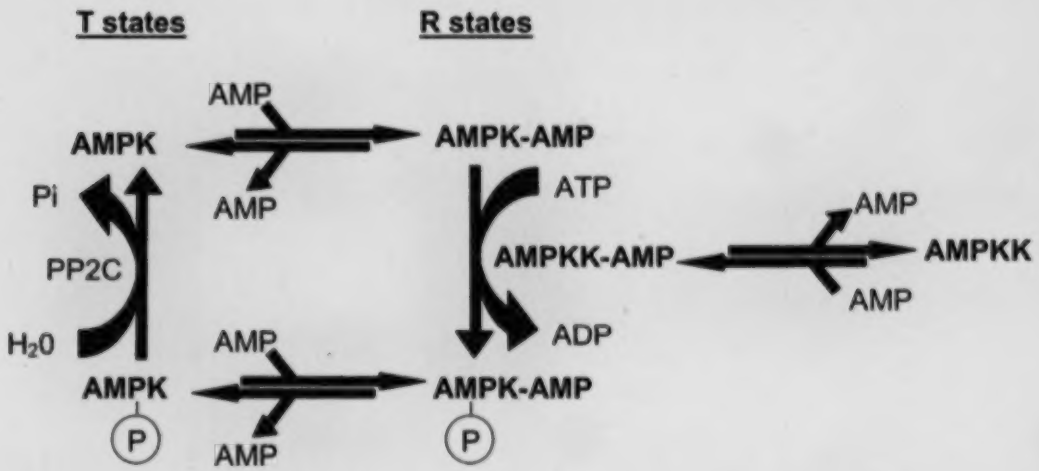

Figure II-3: Model of the transformational states of AMPK (Modeled after Hardie et al., 1998)

II.V Structure of AMPK

Davies et al. (1994), first provided the evidence for the existence of three distinct proteins that constitute the AMPK complex, which are now referred to as the α, β, and γ subunits. The AMPK heterotrimer consists of one catalytic subunit (α) and two regulatory subunits (β and γ). The exact functions of the non-catalytic subunits are presently the focus of investigation. It is known that they are necessary for optimal enzyme activity, playing a key role in stabilizing and uncovering the catalytic site Thr^{172} of the α subunit in the presence of AMP (Dyck et al., 1996) (Figure II-4).

In the inactive form (Figure II-4 A.), the complex exists with no interaction between the α and γ subunits except for the indirect link via the β subunit. In this state, the auto-inhibitory region blocks phosphorylation of Thr^{172} by AMPK-K. When AMP is present (Figure II-4 B.), it binds to residues on both the γ subunit and the auto-inhibitory region, allosterically modifying the complex to uncover the catalytic site, and allow phosphorylation to occur. The β and γ subunit may also play a role in substrate targeting (Gao et al., 1996).

Two major isoforms of the catalytic subunit have been described (α1 and α2), while three isoforms of the β (β1, β2, and β3) and γ (γ1, γ2, and γ3) each with distinct substrate affinities and cellular distribution patterns (Salt et al., 1998; Woods et al., 1996). The α1 subunit seems to be rather ubiquitous, being the predominately expressed isoform in numerous tissues, whereas the

α2 subunit is the predominant isoform expressed specifically in liver, cardiac and skeletal muscle (Stapleton et al., 1996; Cheung et al., 2000).

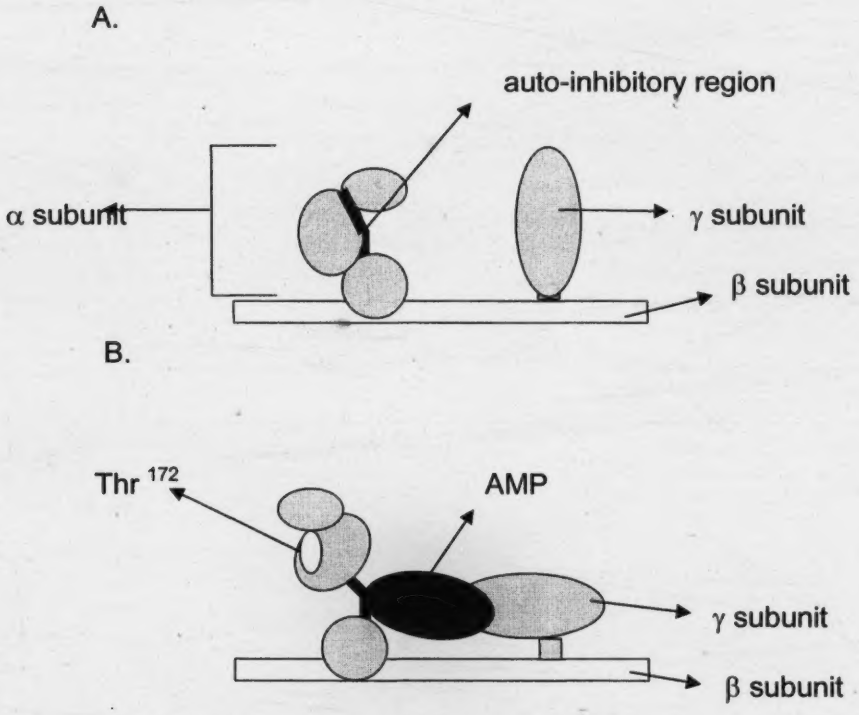

Figure II-4: Model for the Allosteric Modulation of AMPK (adapted from Cheung et al., 2000) in the inactive state (A) and active state (B).

Durante et al., (2002) examined the distribution of the different isoforms in human skeletal muscle and observed that the β2 and γ2 isoforms were most highly expressed in white quadriceps, and γ3 was expressed in red quadriceps and soleus. As well, Salt et al., (1998) found that α2 isoforms in rat hepatocytes exhibited a higher AMP dependence (approximately 3-fold) than

did α1 isoforms in direct allosteric activation and in reactivation by AMPK-K. The same study also used confocal microscopy and Western blotting to examine the subcellular location of each isoform in rat hepatocytes and found the α2, but not the α1, isoform to be located in the nucleus. As the present study was being undertaken, Ai et al., (2002) showed prominent α2-nuclear staining around fast-twitch rat muscle fibres. However, the method used to examine the isoform-specific distribution of AMPK failed to reveal any differences. Despite the limitations of the study, the nuclear location of the α2 subunit is consistent with the hypothesis that the heterotrimeric isoform containing this subunit may regulate phosphorylation of nuclear targets and form the basis for regulating gene expression in response to quantitative or qualitative changes in cellular energy charge.

II.VI Adenine-Independent Activators of AMPK

In addition to disturbances in adenine nucleotide levels, other intermediaries have been shown to affect the activity of AMPK. AICAR is taken up by skeletal muscle, and is phosphorylated by adenosine kinase to form the AMP analogue ZMP. Thus, AICAR administration leads to an accumulation of ZMP, which activates AMPK without affecting the ATP-to-AMP ratio (Corton et al., 1995). As with accumulating AMP levels, ZMP is phosphorylated to ZTP by the action of 5-phosphoribosyl-1-pyrophosphate synthetase (PRPP synthetase) (Sabina et al., 1984). Interestingly, several

studies have found that ZMP accumulates to greater levels in slow- and mixed-twitch muscle homogenates following acute subcutaneous AICAR injections (Buhl et al., 2002; Holmes et al., 1999; Winder et al., 2000).

The anti-diabetic drug metformin has also recently been shown to activate AMPK in intact cells and *in vivo* via an adenine-independent mechanism. Metformin has long been used to treat type 2 diabetes, but its molecular target was unclear until Zhou et al., (2001) found that it activated AMPK. Hawley et al., (2002) were unable to completely establish the mechanism by which metformin activated AMPK, but found AMPK to be phosphorylated at Thr^{172} independent from changes in the ATP-to-ADP ratio, but not by any known AMPK-K. This work suggests that there may be additional upstream kinases capable of activating AMPK without accumulating AMP levels.

II.VI Actions of AMPK

II.VI.i AMPK and Contraction-Induced Skeletal Muscle Glucose Uptake

The uptake of glucose into skeletal muscle is dependant upon the delivery of glucose to the cell and the transport of glucose across the membrane into the cell. Both exercise and insulin induce a rapid increase in the rate of glucose uptake in the contracting skeletal muscles. The concomitant enhanced membrane glucose transport capacity is facilitated by the recruitment of glucose transporters (GLUT4) to the sarcolemma and t-tubules. Although the insulin-mediated pathway is well understood, the

signalling responsible for the contraction-induced regulation of glucose uptake is not yet clear. Several possible mediating agents appear to play a role in the process.

AMPK was first linked to glucose uptake when Merrill et al. (1997), found increased glucose uptake in rat skeletal muscle following AICAR administration. Further support linking AMPK and contraction-induced glucose uptake was based on the findings that AMPK activated by AICAR resulted in insulin-independent glucose transport (Hayashi et al., 1998), and that AICAR and insulin have additive effects on glucose uptake, whereas AICAR and contraction did not (Ai et al., 2002). Secondly, both contraction-induced uptake and AICAR affect glucose transport through a phosphoinosotide 3-kinase-indepedant mechanism (Hayashi et al., 1998). Lastly, a positive relationship was found between the activation of both AMPK and glucose uptake when rodent skeletal muscle was subjected to different cellular stressors including hypoxia, AICAR and electrical stimulation (Hayashi et al., 1998). Collectively, these findings are suggestive of AMPK playing a role in contraction-induced glucose uptake but as yet conclusive evidence has not been produced.

II.VI.ii Role of AMPK in Skeletal Muscle Fatty Acid Oxidation

The movement of fatty acids into the mitochondria is dependent upon a carnitine transport system. Carnitine-palmitoyl transferase-1 (CPT1) is responsible for transporting activated long chain fatty-acids across the outer

mitochondrial membrane, while CPT2 transports them across the inner mitochondrial membrane to be oxidized. At rest, β-oxidation is limited due to the inhibitory action of malonyl-CoA on CPT1 (Ruderman et al., 1999). During exercise however, the levels of cellular malonyl-CoA are decreased (Rassmusen et al., 1997), allowing an increased transport of fatty-acyls into the mitochondria.

The enzyme responsible for the synthesis of malonyl-CoA is acetyl-CoA carboxylase (ACC), which catalyzes the carboxylation of acetyl-CoA producing Malonyl-CoA. Rasmussen et al., 1998 found that during exercise the activity of ACC in skeletal muscle was inhibited. Inhibition of ACC presumably results in reduced cellular Malonyl-CoA levels, subsequently relieving inhibition of CPT1. The transport of fatty acids into the mitochondrial matrix is then increased, up-regulating the rate of flux through the β-oxidation pathway.

The skeletal muscle isoform of ACC, isolated from rat hindlimbs, was shown to be phosphorylated by both cAMP-dependant-protein kinase and AMPK in vitro, but only phosphorylation by AMPK completely inactivated ACC (Winder et al., 1996). This finding was further substantiated by studies revealing ACC is inactivated concurrently with AMPK activation in rats exercising on the treadmill (Rasmussen et al., 1997), in electronically stimulated muscle (Hutber et al., 1997; Vavvas et al., 1997), and in resting perfused muscle incubated with AICAR (Merril et al., 1997). As such, AMPK plays a key role in attenuating the rate of malonyl-CoA synthesis by phosphorylating and inactivating ACC.

However, in addition to the role AMPK plays inhibiting the rate of malonyl Co-A synthesis, it has also been linked to the reduction of malonyl Co-A concentrations seen during exercise. The enzyme malonyl-CoA decarboxylase (MCD) is responsible for the degradation of malonyl-CoA by cleaving one carbon, and producing acetyl-CoA. Saha et al. (2000) found MCD activity to be increased in both electrically stimulated rat gastrocnemius muscle and AICAR treated rat extensor digitorum longus (EDL) muscle, however, this result is somewhat controversial. The stimulated activation was removed from both conditions following treatment with protein phosphatase 2A, providing evidence that phosphorylation is involved. While this study does much to link AMPK to the contraction-induced the activation of MCD in rat skeletal muscle, no direct evidence has been found.

Interestingly, Winder et al. (1990) found that malonyl-CoA levels dropped rapidly in type IIA fibres, but not in IIB, suggesting a fibre-type specific regulation of malonyl-CoA in rat muscle. Decreased malonyl-CoA concentrations seen in IIA fibres following contractile activity could be due to greater ACC activity, as Type IIA fibres utilize the β-oxidative pathway to a much greater extent than do IIB fibres.

While the aforementioned studies are beginning to uncover the role AMPK plays in regulating fatty acid oxidation in rodents, its role in human muscle is less clear. Concentrations of malonyl-CoA are much lower in human muscle, and as such are more difficult to measure (Winder, 2001). In

absence of conclusive evidence, it seems logical that AMPK regulates fatty acid oxidation in human skeletal muscle as well.

II.VI.iii AMPK and Contractile Activity in Skeletal Muscle

Vavvas et al., (1997) found that following 5 minutes of electrically induced contraction of rat hind limb muscles, the $\alpha 2$ isoform accounted for 90% of total AMPK activity and remained elevated for 90 minutes upon cessation of contraction. In contrast, the $\alpha 1$ isoform was responsible for only 10% of the total activity during stimulation and was inactivated during the entire post-stimulation period. These findings were confirmed with human skeletal muscle samples following 60 minutes of exercise at 75% of VO_2max (Fujii et al., 2000; Wojtaszewski et al., 2000). Also of interest is the fact that the $\alpha 2$-specific activity remained elevated above basal levels for 3-hours during the post-exercise recovery period. This post exercise activity may be an acute signal linked to the phenotypic adaptive response to the stress of the exercise bout.

An interesting study by Durante et al., (2002) observed the effects of endurance training on activity and expression of AMPK in rat red (mixed-twitch) and white (fast-twitch) quadriceps and soleus (slow-twitch) muscle. Moderate treadmill exercise was shown to elicit a large activation of $\alpha 2$-AMPK in the red quadriceps, a small activation of $\alpha 2$-AMPK in soleus and no

activation of AMPK in white quadriceps. However, these authors noted that the moderate intensity of exercise used would primarily recruit the soleus and red quadriceps, and not be of a high enough intensity to recruit and activate AMPK in white quadriceps. Activation of α1-AMPK was negligible in all three types of muscle following both acute exercise and 14 weeks of training.

After training however, a greatly reduced activation of α2-AMPK in red quadriceps, due to a reduction of metabolic stress, no change in soleus α2-AMPK activity, and a small increase in activity of α2-AMPK due to phosphorylation of Thr^{172} were observed. Expression of the α and β subunits were unchanged post-training, however, there was a three-fold increase in the expression of the γ3-AMPK isoform in the red quadriceps. Durante et al., (2002) provided novel data to the role AMPK plays during moderate exercise, however this study failed to maximally activate AMPK and to compare AMPK isoform-specific expression between the various muscle types.

II.VI.iv AMPK and Gene Expression

Research into AMPK has recently branched from its molecular roles within the cell to focus on the ability to alter gene expression. Early hypotheses of this nature were fuelled by the plant homologue of AMPK. The budding yeast *Saccharomyces Cerevisae* has a similar protein kinase complex that acts in times of cellular stress. *S. Cerevisae* uses glucose as its preferred carbon source, and genes involved in the metabolism of alternative glucose

sources are repressed when glucose is available (Hardie et al., 1998). The sucrose non-fermenting (SnF) system is activated when glucose is removed as a substrate, and plays a key role in de-repression of the aforementioned genes through phosphorylation of transcription factors (Celenza & Carlson, 1986). SnF1 protein kinase and AMPK are both activated in times of cellular stress, share a similar method of regulation, downstream targets, and amino acid sequence around the catalytic subunit (Hardie et al., 1998). Therefore it seems reasonable that AMPK may also be involved in phosphorylating transcription factors.

Winder et al. (2000) investigated the effect of chronic activation of AMPK on mitochondrial enzymes in skeletal muscles of rats. AMPK was activated pharmacologically with AICAR. Following 28 days of 1mg/g (of body weight) AICAR injections, they found a significant increase in the activity of the mitochondrial citric acid cycle enzymes citrate synthase, succinate dehydrogenase, and malate dehydrogenase in white quadriceps and soleus. As well, previous authors have shown AMPK is capable of decreasing the transcription of the gluconeogenic enzymes phosphoenolpyruvate carboxykinase and glucose-6-phosphatase in liver (Lochhead et al., 2000). AMPK has also been shown to inhibit the transcription of fatty acid synthase, L-type pyruvate kinase and the lipogenic gene, Spot 14 in hepatocytes. (Reviewed by Hue et al., 2002). These data indeed suggest that AMPK may also play a secondary cellular role, directly or indirectly, in regulating gene

transcription in response to changing environmental demands that lead to changes in the cellular energy charge.

II.VII Summary

AMPK is a heterotrimeric (Davies et al., 1994) member of a highly conserved cascade that responds to minute perturbations in the AMP-to-ATP ratio within cells (Winder et al., 2001). Once activated, via a complex process of regulation involving allosteric and covalent modification (Hardie et al., 1998, Kemp et al., 1999), AMPK phosphorylates multiple metabolic targets that serve to increase ATP production via increased fatty acid oxidation (Winder et al., 1996; Rasmussen et al., 1997; Hutber et al., 1997; Vavvas et al., 1997; Merril et al., 1997) and increased glucose uptake (Merrill et al., 1997;Hayashi et al., 1998). AMPK has also been linked to playing a role in signalling similar adaptations that occur following endurance exercise training such as increased mitochondrial biogenesis (Bergeron et al., 2001) and increased oxidative enzymes (Winder, 2000). However, the mechanisms for these actions are still unclear.

Chapter III

Methods

III.I Animals

Three to four month-old (400-600g), male Sprague-Dawley rats (n=6) were obtained from the local breeding colony at Health Sciences Laboratory Animal Services at the University of Alberta. They were housed in a thermally controlled room maintained at 22 °C on 12:12-hour light-dark cycles and fed and watered *ad libitum*. All animal experiments received ethics approval from the Animal Welfare Committee at the University of Alberta and were completed in accordance with the requirements of the Canadian Council on Animal Care. Six animals were sequestered for AICAR administration, and six others were used for immunohistochemical analysis.

III.II AICAR Injection

Animals received an acute subcutaneous injection (0.5 mg/g body weight) of 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR) (Sigma-Aldrich, St. Louis, MO) in sterile phosphate buffered saline (PBS) two hours before being euthanized by CO_2 asphyxiation. The animals were exanguinated prior to muscle extraction and mean extraction time was 13.3±3.5 minutes (from the start of asphyxiation to the completion of muscle extraction) (n=6). At the dosage used in this experiment, AICAR has been

shown to be a powerful activator of AMPK in rat muscle (Corton et al., 1995; Winder et al., 2000).

III.III Tissue Handling

The soleus and gastrocnemius muscles were dissected from the right hind leg of the euthanized rats and immediately flash-frozen in liquid nitrogen, while the same muscles dissected from the contralateral hind leg were frozen in isopentane cooled to its melting point (-159 °C) by liquid nitrogen, for immunohistochemical analysis. The red and white portions of the flash-frozen gastrocnemius muscles were isolated in a cryostat at -20 °C, then powdered under liquid nitrogen, and freed of blood and connective tissue for subsequent analysis.

III.IV Tissue Homogenization and PEG Precipitation

Muscles were manually homogenized in four-volumes of an ice-cold lysis buffer containing the following: 0.05 M Tris, 0.25 M Mannitol, 1 mM EDTA, 1 mM EGTA, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 10% w/v glycerol, 1% Triton X-100, 1 mM DTT, 0.1% of a mammalian protease inhibitor cocktail (Sigma) [i.e., 4-(2-aminoathyl) benzensulfonyl fluoride (AEBSF), pepstatin A, trans-epoxysuccinyl-L-leucyl-amido(4-guanidino)butane (E-64), leupeptin, and aprotinin]. Homogenates were centrifuged at 4°C for 20 minutes at 14,000×g and the resulting supernatant diluted 10-fold in a 25% solution of polyethylene glycol 6000 (PEG 6000). The

solution was vortexed for 5 minutes and then centrifuged at 10 000×g for 10 minutes at 4°C. The resulting supernatant was collected and made up to 6% PEG 6000 with 25% PEG 6000, vortexed, and centrifuged as before. The resulting pellet was washed in a 6% PEG solution and dissolved in a resuspension buffer containing 0.1 M Tris-base (pH 7.5), 1 mM EDTA, 1 mM EGTA, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.1% protease inhibitor cocktail (Sigma), and 10% glycerol (w/v). Protein concentrations were determined by the Lowry method (Lowry et al., 1951).

III.V Assay of Total AMPK Activity

AMPK activity was assayed in rat soleus and red and white gastrocnemius extracts according to Kudo et al. (1995). Total AMPK activity was assayed on 1 mg/ml whole muscle extracts purified by PEG precipitation by measuring the incorporation of γ-^{32}P-ATP into AMARA peptide (AMARAASSAAALARRR, API). The reaction was initiated by adding 5 μg of sample to 20 μl of an assay buffer containing, 80 mM HEPES, 160 mM sodium chloride, 1.6 mM EDTA, 16% glycerol, 1 mM AMARA peptide, 1 mM AMP, 0.8 mM DTT, 5 mM $MgCl_2$, 1 mM γ-^{32}P-ATP, 0.01% Triton X-100, and 40 mM Hepes-NaOH, pH 7.0. Non-specific activity was determined by replacing samples with the assay buffer. After 5 minutes at 30°C, 15 μl of the reaction mixture was removed, spotted onto 1.5×1.5 cm phosphocellulose paper (Whatman, P81) and 2-3 seconds later stirred in a solution of 1% (v/v)

phosphoric acid to stop the kinase reaction. The phosphocellulose papers were then washed an additional four times in the phosphoric acid and once in acetone. After drying, the papers were placed in 4 ml of Optiscint "Hisafe" liquid scintillation fluid and radioactivity was determined using standard beta counting procedures. Specific radioactivity was measured on 5 μl of γ-^{32}P-ATP added to scintillation fluid. Triplicate preparations included "no AMP/no AMARA", "no AMP/with AMARA" and "with AMP/with AMARA" conditions to account for basal phosphorylation levels within the samples. Specific total AMPK activity was expressed in pmoles·minute^{-1}·mg protein^{-1}.

III.VI Preparation of Recombinant Full Length α1- and α2-Catalytic Subunits of AMPK

Full-length cDNAs were ligated into pCR2.1-TOPO cloning vectors and transformed into chemically competent *E.Coli*. Transformants were selected for kanamycin resistance, according to the manufacturer's instructions (Invitrogen). Purified plasmid DNA was subsequently isolated using the SNAP MiniPrep Kit (Invitrogen), and analyzed for size and orientation by restriction analysis using EcoRI (α-1 and α-2), and KpnI (α-1) or BamHI (α-2). The correct orientation and sequences of plasmid inserts were confirmed by independent sequence analysis (DNA Sequencing Laboratory, University of Alberta). Recombinant plasmids were purified (Plasmid Midi Purification Kit, QIAGEN) and the inserts removed by sequential digestion with KpnI and XhoI,

followed by purification (QIAquick, QIAGEN), visualization by agarose gel electrophoresis and elution of insert bands (GenElute™, Sigma). The eluted bands were subsequently ligated into the pET30a(+) expression vector (Novagen), sequenced (Molecular Biology Services Unit, University of Alberta) and transformed into DH5a competent cells to generate plasmid copies (Life Technologies). Plasmids were purified, quantified, and used to transform the expression strain of Escherichia coli, BL21(DE3). Transformants were selected according to manufacturer's instructions and stored at –80 °C until use.

Large-scale expression of target DNA was induced by 2-hr exposure of the expression transformants to 1 mM isopropyl-B-D-thiogalactopyranoside (IPTG) in the presence of 1% glucose. The recombinant His-tagged AMPK a-subunits were released from lysed bacteria, and cleared by centrifugation (12,000xg, 4C) and filtration. The filtrates were applied to Ni2+ charged HiTrap affinity columns
(Amersham Pharmacia) and step-eluted with imidazole. Fractions were stored at –80 °C until use.

III.VII Western Blot Analysis

Purified α1- or α2-recombinant proteins (produced in our laboratory according to the aforementioned procedure) and tissue samples were denatured in reducing sample buffer and heated for 5 min at 95°C; 5μg of purified protein or 40μg of total tissue protein were loaded per lane. Proteins

were separated on a 9% polyacrylamide gel at 115 volts (BioRad Mini-PROTEAN 3). Proteins were then transferred onto Immuno-Blot PVDF membrane (BioRad) at 4°C for 60 minutes; transfer efficiency and loading were confirmed by Ponceau-S staining. After de-staining, the membrane was blocked overnight at 4°C in TBS containing 2.5% fat-free dry milk powder, 1% BSA and 0.1% Tween-20. After repeated washes in TBS-Tween (0.1%), membranes were incubated overnight at 4°C in anti-alpha-1 (i.e., 1:500) or anti-alpha–2 (i.e., 1:500) AMPK IgG antibodies (Upstate Biotechnologies, USA) diluted in blocking buffer. Horseradish peroxidase (HRP)-conjugated goat-anti-rabbit IgG secondary antibody (i.e., 1:1000 in blocking solution) (Vector Laboratories, Canada) was applied to the membranes for 90 minutes at room temperature. Immunoreactivity was localized using enhanced chemiluminecence (ECL-Western Blot detection reagent, Amersham, U.K.). All blots were evaluated by integrating densitometry (ChemiGenius and Gene Tools, SynGene U.K.).

III.VIII Nuclear Isolation from Skeletal Muscle

Nuclear and extra-nuclear fractions were isolated according to Neufer et al. (1993) and Zahradka et al. (1989). Eight grams each of gastrocnemius and soleus muscle was minced and homogenized (Polytron, setting 5-6) in an ice-cold lysis buffer (10 mM HEPES, 5 mM KCl, 10 mM $MgCl_2$, 10 mM EDTA, 5 mM β-ME, 1:1000 mammalian protease inhibitor cocktail (Sigma), pH 7.5) containing 0.32 M sucrose. The resulting homogenate was then filtered

through cheesecloth and centrifuged at 1000 g for 8 minutes at 4°C. The supernatant was decanted and the pellet was resuspended in 35ml of the lysis buffer containing 2.2 M sucrose before a second spin at 80 000g for 90 minutes at 4°C.

Following the high-speed centrifugation, the resulting fluffy white pellet was extracted and resuspended in 2ml of sterile storage buffer (75 mM HEPES pH 7.5, 60 mM KCl, 15 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, 0.5 mM Spermine, 0.5 mM Spermidine, 40% glycerol).

The nuclei were then washed and collected by centrifugation at 5000g for 10 minutes at 4°C. Pellets were resuspended in 200µl of storage buffer. Confirmation of nuclear isolation was obtained by staining fractions with Trypan Blue. Western blotting analysis (described above) was used to quantify the nuclear and non-nuclear fractions (selected as the supernatant with a determinable protein concentration).

III.IX Immunohistochemistry for Alpha-1 and Alpha-2 AMPK Isoforms

Twelve µm thick serial sections of rat medial gastrocnemius and soleus were air-dried. Pilot experiments revealed that the method with no fixation was optimal for antigen retrieval (evidenced by the darkest staining). Sections were washed once in PBS-Tween, twice in PBS, and incubated for 30 minutes in 3% H_2O_2 in methanol, washed again and incubated overnight at 4°C in the blocking solution containing, 1% BSA, 5% goat serum in PBS-Tween (0.1%).

Sections were then washed in PBS and incubated in succession with Avidin-D Blocking Solution (Vector Laboratories), Biotin Blocking Solution (Vector Laboratories) and PBS. Primary anti-α1-AMPK or anti-α2-AMPK were diluted in the blocking solution (1:60, Upstate Biotechnologies) and applied to sections for 1 hr. Sections were then washed once in PBS-Tween, twice in PBS, and reacted for 30 min with biotinylated goat-anti-rabbit-IgG (1:400) (Vector Laboratories). After washing, sections were incubated for 60 minutes with biotin-avidin-HRP, washed and reacted with the substrate solution containing DAB, H_2O_2, and $NiCl_2$ in 50 mM Tris, pH 7.5 (Vector Laboratories). The reaction was stopped after 6 minutes by rinsing with distilled water. Sections were subsequently dehydrated in successive rinses of 50%, 70%, and 3 x 100% ethanol, cleared by three separate rinses in xylene and mounted in Entellan (Merck, New Jersey, USA).

III.X Myosin Heavy Chain Determination - SDS-PAGE

MHC isoforms were first assessed quantitatively via electrophoresis (SDS-PAGE) (Talmadge and Roy, 1993) in white and red gastrocnemius and soleus. Briefly, samples were pulverized under liquid nitrogen and prepared according to the method of Laemmli (1970) in a 0.4 M Tris solution also containing 1.84% sodium-dodecyl sulfate (w/v), 4% 2-mercaptoethanol (v/v) and 6.4% glycerol (v/v). After heating, the samples were alkylated via the addition of 10 μl of 20% iodoacetamide. One μg of protein was loaded into each lane of the stacking gel composed of 25% glycerol and 4% acrylamide.

Samples were separated on a 7% PAA (polyacrylamide) separating gel containing 30% glycerol at a constant voltage of 275 volts for 24 hours at 10 °C (Hoefer SE600, Amersham Biosciences). Silver staining was performed according to the method of Oakley et al., (1980) III.IV. All gels were documented and analyzed densitometrically with the Syngene ChemiGenius bio imaging system using Genesnap and Genetools analysis software (Syngene, U.K.).

III.XI Myosin Heavy Chain Immunohistochemistry

III.XI.i Antibodies

To examine the isoform specific distribution of AMPK according to individual fibre types, the following monoclonal antibodies directed against adult myosin heavy chain (MHC) isoforms were used: MHCI [NOQ7.5.4D Harris et al., 1989}; MHCIIa [SC-71 (Schiaffino et al., 1989)], and MHCIIb [BF-F3 (Schiaffino et al., 1989)]. Non-specific control mouse-IgG and rabbit-IgG antibodies were obtained from Santa Cruz Biochemical.

III.XI.ii Immunohistochemical Staining

Melting isopentane frozen medial gastrocnemius and soleus muscle sections were cut and applied to poly-L-lysine slides purchased from Cedarlane Laboratories (Hornby, ON, Canada). Sections were washed PBS and PBS-tween and incubated with 3% H_2O_2 in methanol for 30 min. Blocking solutions were prepared in PBS-tween with 1% BSA and 10% horse (BS-1) or

goat serum (BS-2) and sections were incubated overnight at 4 °C. Slides were washed as above and antibodies were diluted in either BS-1: NOQ7.5.4D (Harris et al., 1989) (MHC I, 1:4000); SC-71 (MHC IIa, culture supernatant, 1:50); IgG control (non-selective IgG, 1:2000) or in BS-2: BF-F3 (MHC IIb, culture supernatant, 1:50) and applied for 90 minutes at room temperature (Schiaffino et. al., 1989). Slides were washed again before application of secondary biotinylated antibodies diluted 1:400 in appropriate blocking solution. Anti-mouse-IgM/BS-2 was applied to slides that had received the BF-F3 antibody while all others were incubated with anti-mouse-IgG/BS-1. Biotinylated secondary antibodies (Vector Laboratories) were applied in BS-1 or BS-2 for 60 minutes at room temperature. Sections were incubated with Vectastain ABC reagent (Vector Laboratories) for 30 minutes at room temperature and immunoreactivity was visualized with a solution of diaminobenzidine/$NiCl_2$ applied for 6 minutes (Vector Laboratories). This array of monoclonal antibodies allowed direct identification of fibres expressing Type I, IIa and IIb MHC, while MHC IId was considered present in all fibres not staining for at least one of the other adult isoforms.

III.XII Statistical Analyses

Data are presented as mean ± SEM except were noted. AMPK activity was compared using a 3 (muscle types) x 1 (activity) ANOVA and immunohistochemical AMPK isoform-specific expression was analyzed using a 3 (isoform conditions) x 4 (fibre type) ANOVA. When significance was found

between groups, a Tukey post-hoc test was used. For all analyses, the α-level was set *a priori* at 0.05.

Chapter IV

Results

IV.I Fibre-Typing of Rat Skeletal Muscles

Analysis of the MHC expression of the slow-, fast-, and intermediate-twitch muscle fibres by SDS-PAGE revealed that the soleus (SOL) was composed of slow MHC-I & -II, while white gastrocnemius (WG) consisted of fast MHC-IIb & -IId/x. In contrast, red gastrocnemius (RG) contained all four MHC isoforms (Figure IV-1A and B). While the SOL contained slow MHC-I & -II, it was MHC-I that was predominately expressed, displaying a four-fold greater content than MHC-II when analyzed by densitometry (Figure IV-1A). Similarly, WG revealed more than a four-fold greater expression of MHC-IIb than the co-expressed MHC-IId/x isoform. Lastly, while expressing all four MHC isoforms, the intermediate-twitch RG exhibited a MHC profile leaning toward the fast end of the fibre-type continuum, with close to one third of the total MHC content being comprised by MHC-IIb and -IId/x.

Patterns of MHC expression are shown in Figure IV-2 for medial gastrocnemius (MG) and soleus (SOL). The results showed that the MG contained all four MHC isoforms, with approximately 60% of its total being comprised of fast MHC-IIb, while the SOL expressed predominately MHC-I (Figure IV-3). Fibres not staining positive for any of MHC-I, -IIa, or -IIb, were delineated as type-IID(X) fibres.

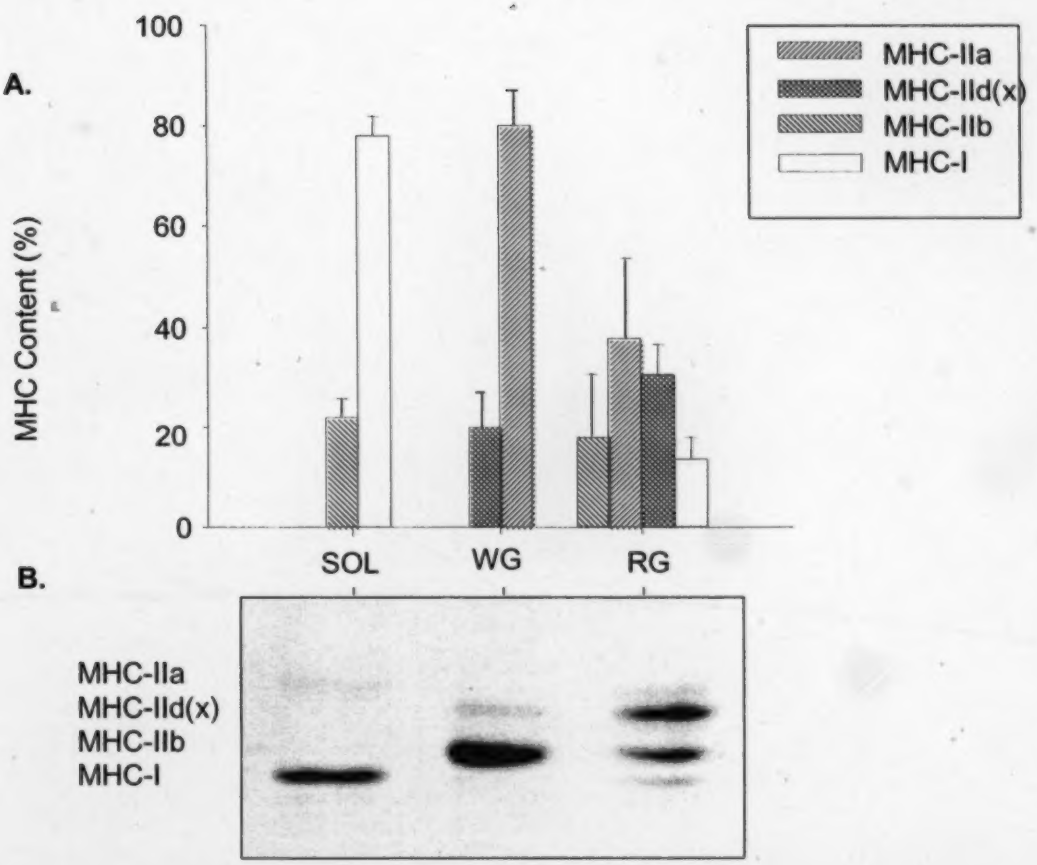

Figure IV-1: MHC Expression in soleus (SOL), white (WG) and red gastrocnemius (RG) analysed by SDS-PAGE. **A.** Data expressed as a percentage of the total MHC content for each muscle and are means ± SEM, for each muscle *n*= 6. **B.** Representative section from the gel displaying the bands and their corresponding MHC isoform for the three respective muscles.

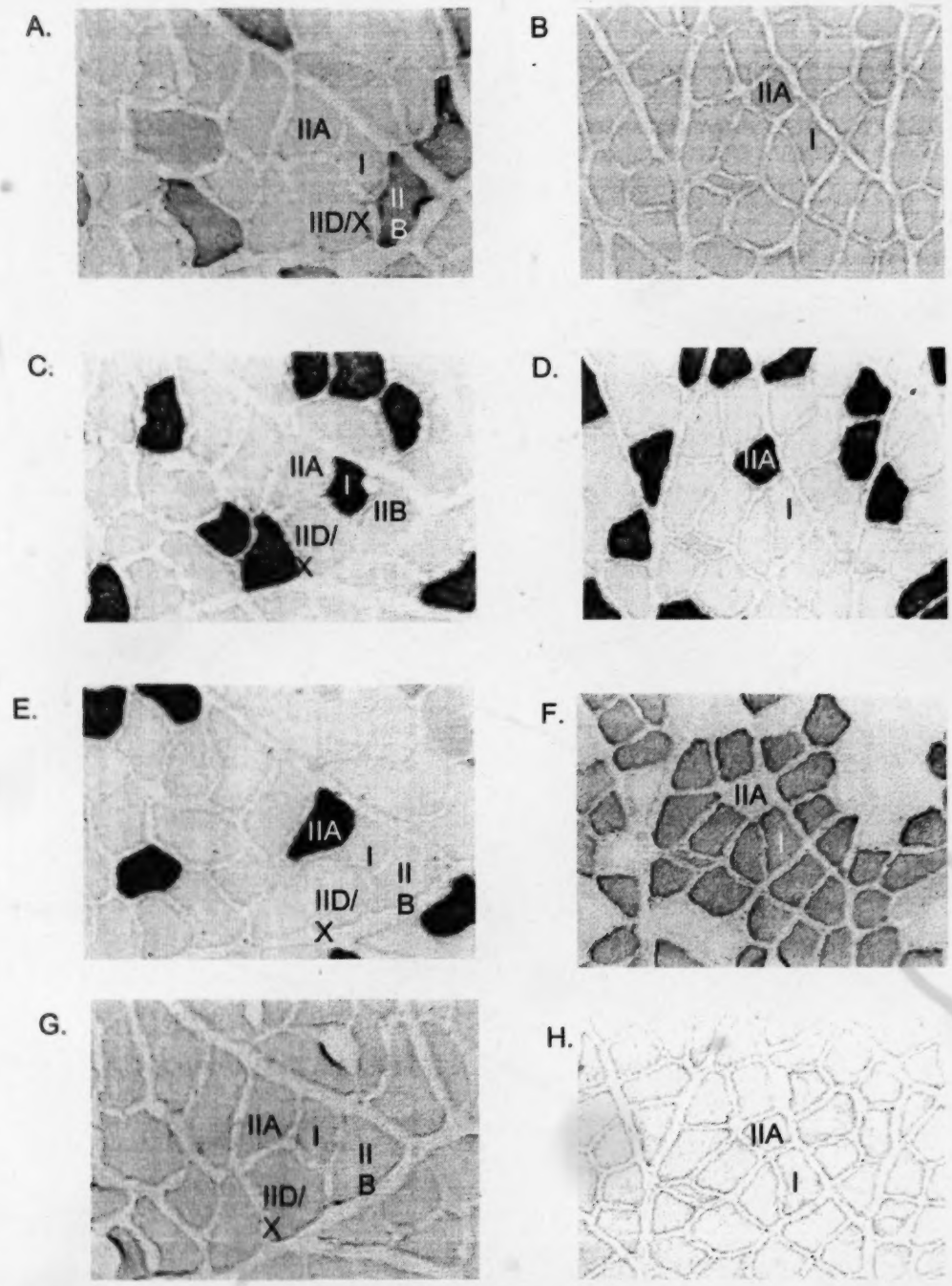

Figure IV-2: Immunohistochemical fibre type distribution. Representative staining patterns for fibre type distribution on serial sections of medial gastrocnemius (A,C,E,G) and soleus (B,D,F,H) stained for: A&B MHC-IIb(BFF-3), C&D MHC- I(BAD-5), E&F MHC-IIa(SC-71), G&H Control (IgG).

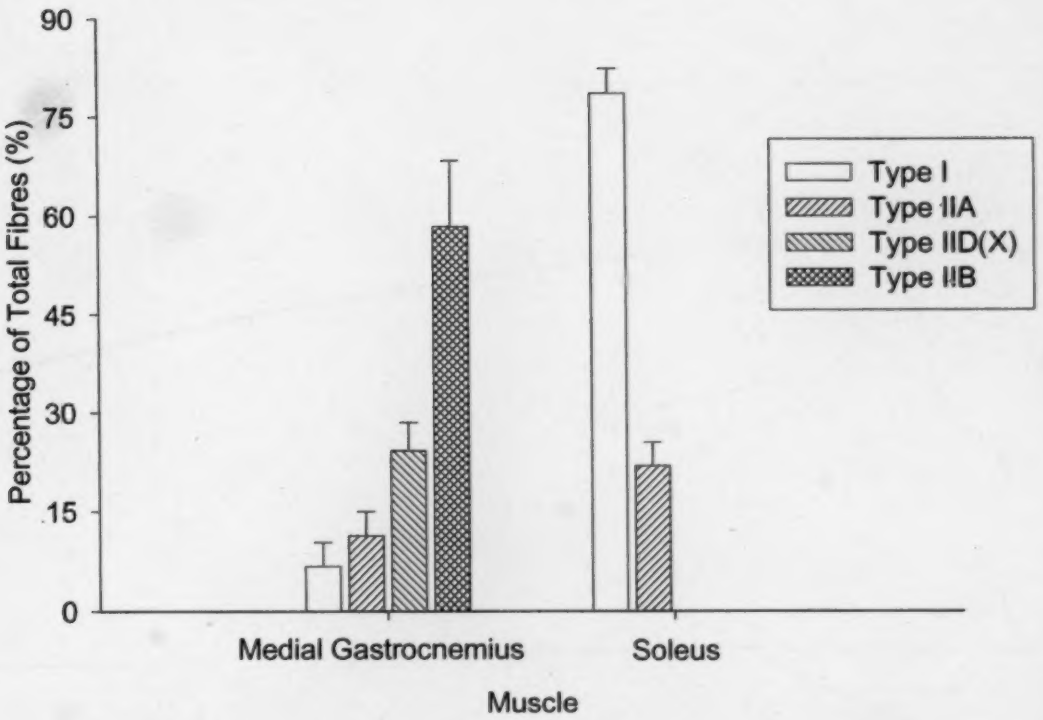

Figure IV-3: A comparison of fibre type distribution in medial gastrocnemius (MG) and soleus (SOL) muscles. Analyzed by immunohistochemistry and expressed as a percentage of total fibres analysed per muscle. Data are means ± SEM; MG: n = 6 (mean number of fibres analysed per muscle = 297.3), SOL: n = 4 (mean number of fibres analysed per muscle = 251.5).

IV.II Total AMPK Activity is Highest in Fast Twitch Fibres

Following acute AICAR administration (0.5mg/g) and PEG purification, white gastrocnemius yielded the highest total AMPK activity with a more than two-fold greater activity than either the red gastrocnemius or the soleus (Figure IV-4).

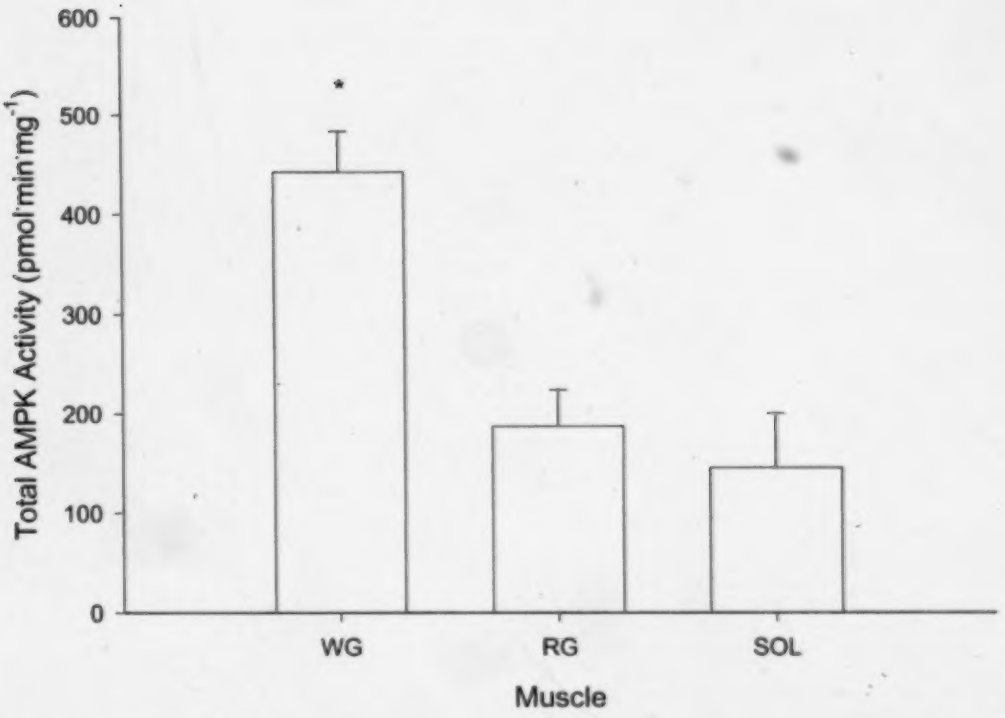

Figure IV-4: Total activity of AMPK in muscles composed of different fibre-types. Activity was measured by determining the rate of incorporation of ^{32}P-γ-ATP into AMARA peptide following PEG purification in white gastrocnemius (WG), red gastrocnemius (RG) and soleus (SOL) muscles. Data are means ± SEM; n = 18, * = $P < 0.05$.

IV.III Isoform-specific AMPK Content in Gastrocnemius and Soleus

Immunohistochemical analysis of the isoform-specific expression of AMPK revealed a fibre-type dependent distribution with the fast medial gastrocnemius and slow soleus (Figure IV-5). Type I fibres in both the soleus and the medial gastrocnemius expressed only alpha-1 AMPK, type-IIA fibres almost exclusively co-expressed both alpha-1 and -2 AMPK, type IID(X) fibres either expressed alpha-2 alone or both alpha-1 and -2, and lastly, the majority

of the fast type IIB fibres expressed only alpha-2 AMPK (Figure 4-5). Interestingly, qualitative analysis revealed two distinct staining intensities prevalent on the medial gastrocnemius stained for α2-AMPK (Figure 4-5 B). Type IID/X and type IIA fibres stained the darkest, while the type IIB stained positively, but not quite as dark as the IID/X and IIA fibres, while the type I fibres showed no staining.

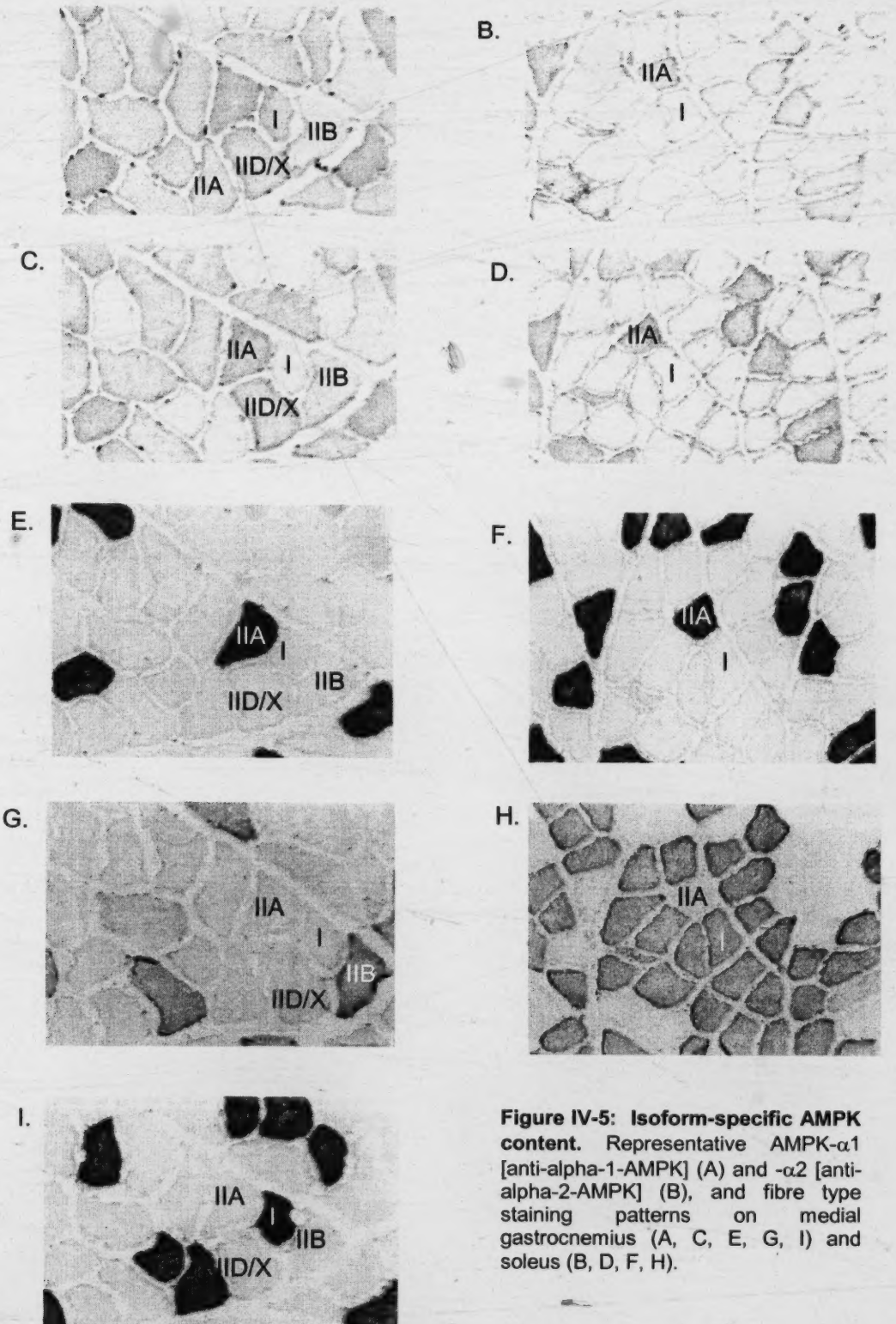

Figure IV-5: Isoform-specific AMPK content. Representative AMPK-α1 [anti-alpha-1-AMPK] (A) and -α2 [anti-alpha-2-AMPK] (B), and fibre type staining patterns on medial gastrocnemius (A, C, E, G, I) and soleus (B, D, F, H).

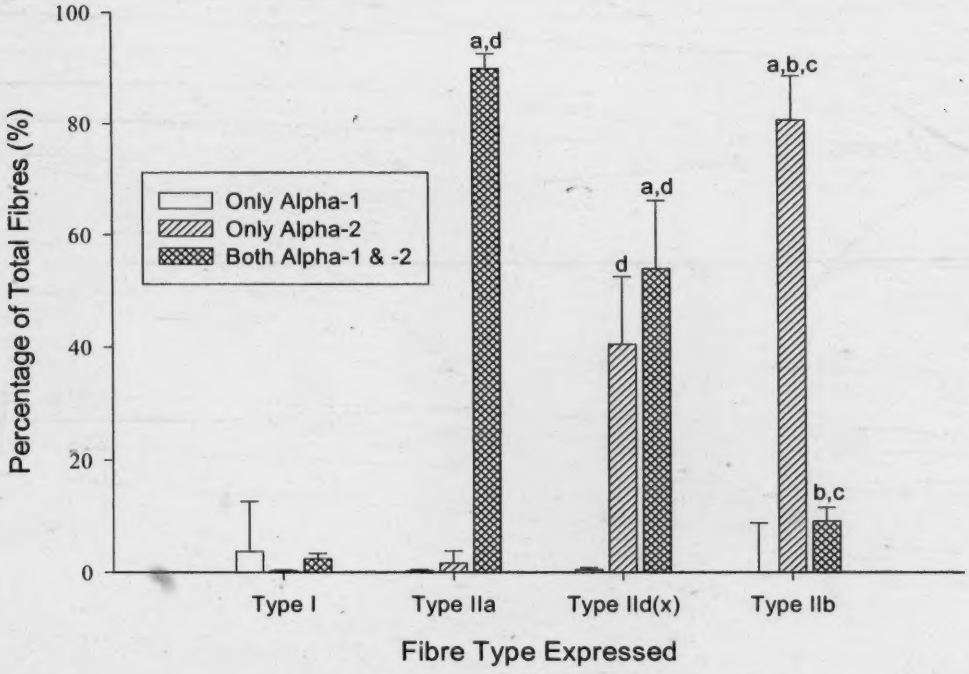

Figure IV-6: Isoform specific distribution of AMP-Activated Protein Kinase (AMPK) in various fibre-types from soleus and gastrocnemius muscle. Expressed as a percentage of total fibres analysed. Data are means ± SEM; n = 10 (mean number of fibres analysed per muscle = 293). [a] significantly different from type I, [b] type IIA, [c] type IID(X), [d] type IIB ($P < 0.05$).

A subsequent analysis was performed comparing the distribution of the two alpha isoforms of AMPK in the same fibre-types across the predominately slow-twitch (soleus) and fast-twitch muscles (medial gastrocnemius). These results revealed no significant differences across the soleus and medial gastrocnemius in fibre-types shared by both muscles (Figures IV-7 and IV-8).

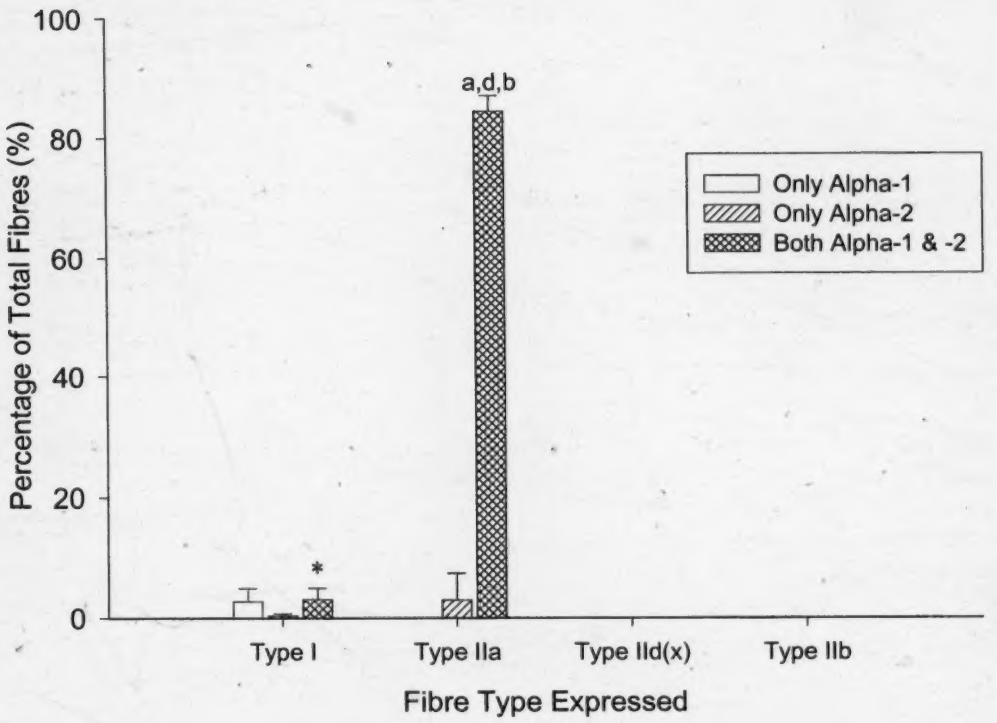

Figure IV-7: Isoform specific distribution of AMP-Activated Protein Kinase (AMPK) in various fibre-types obtained from soleus muscle. Expressed as a percentage of total fibres analyzed. Data are means ± SEM; n = 4 (mean number of fibres analyzed per muscle = 287.5) [a] significantly different from type I, [b] type IIA, [c] type IID(X), [d] type IIB ($P < 0.05$).

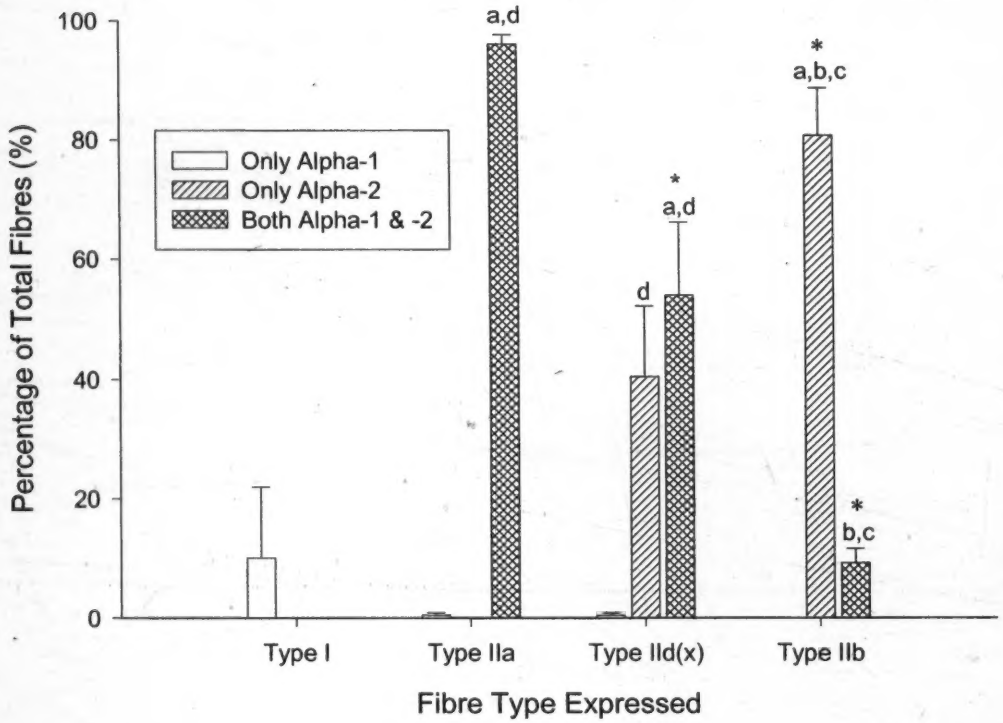

Figure IV-8: Isoform specific distribution of AMP-Activated Protein Kinase (AMPK) in various fibre-types obtained from medial gastrocnemius muscle. Expressed as a percentage of total fibres analyzed. Data are means ± SEM; n = 6 (mean number of fibres analyzed per muscle = 297). [a] significantly different from type I, [b] type IIA, [c] type IID(X), [d] type IIB ($P < 0.05$).

Densitometric analysis of Western blot data (Fig. IV-9) revealed a fibre-type dependent pattern of distribution of the α-1-AMPK and α-2-AMPK isoforms. α-1 content was just slightly greater in the red gastrocnemius than it was in the white gastrocnemius, and lowest in the slow-twitch soleus. Similarly, α-2 content was smallest in the slow-twitch soleus, greater in the red

gastrocnemius and greatest in the fast-twitch white gastrocnemius Total AMPK content was greatest in the white gastrocnemius, second greatest in the red gastrocnemius and least in the soleus (Table IV-1).

A unique double banding was seen on both the both blots with the same pattern being displayed; the white gastrocnemius showed a strong upper band (joined by a faint lower band on the α-1 blot), the red gastrocnemius showed both bands, while the soleus displayed only the lower band. The rAMPK bands showed a smeared appearance, but the most prominent band was located at 63 kDa and coincided with the weight of the lower bands on both blots (Figure IV-9).

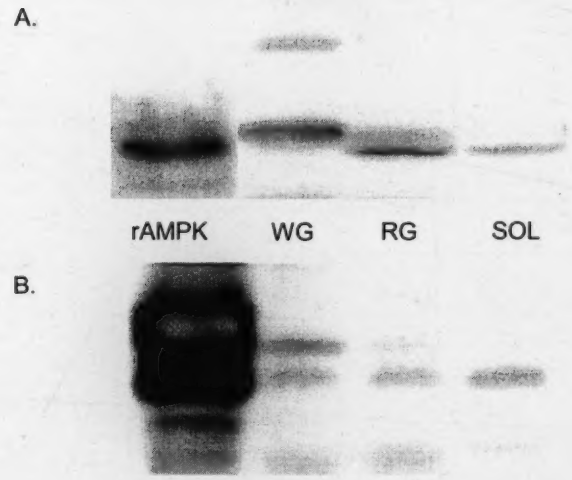

Figure IV-9: The Isoform-specific expression of AMPK in different fibre-types. Representative western blots probed with **A.** anti-alpha-1 and **B.** anti-alpha-2 AMPK antibodies. Recombinant alpha-1 and -2 proteins were used as standard and internal controls.

Table IV-1: Densitometric analyses of isoform-specific AMPK expression. Data are from western blots and are expressed as arbitrary densitometric units ±SEM.

Subunit Isoform	WG	RG	SOL
α1	$2.45 \times 10^7 \pm 0.15$	$2.51 \times 10^7 \pm 0.21$	$3.67 \times 10^5 \pm 0.08$
α2	$3.10 \times 10^7 \pm 0.12$	$2.09 \times 10^7 \pm 0.24$	$1.83 \times 10^7 \pm 0.09$

IV.V Isoform-Specific Subcellular Localization of AMPK in Fast- and Slow-Twitch Muscle

Immunohistochemical analyses revealed nuclei stained positive with anti-alpha2 AMPK polyclonal antibody. By comparison, no staining was observed with anti-alpha1 AMPK (Figure IV-5 A) when compared to control nuclei or those treated with alpha-1 (Figure IV-5 C). Examination by light microscopy showed nuclei positively stained for alpha-1 or -2 in the slow-twitch soleus was similar to that of the alpha-1 stained nuclei in the medial gastrocnemius. However, further analysis revealed a six-fold larger area (with nuclei positively stained for alpha-2) in the fast-twitch medial gastrocnemius when compared to the slow-twitch soleus (Fig. IV-10).

These results were similar to those revealed by our Western Blot analysis of the isoform-specific content of AMPK in the nuclear and non-nuclear fractions from medial gastrocnemius and soleus. Very faint bands can be seen in the nuclear fractions of the soleus stained for alpha-1, and in the extra-

nuclear fraction of the medial gastrocnemius. In contrast, a very prominent band can be seen in the nuclear fraction of the medial gastrocnemius (Fig IV-11). Densitometric analysis confirms the strong nuclear localization of the alpha-2 isoform in the fast-twitch muscle (Fig. IV-12).

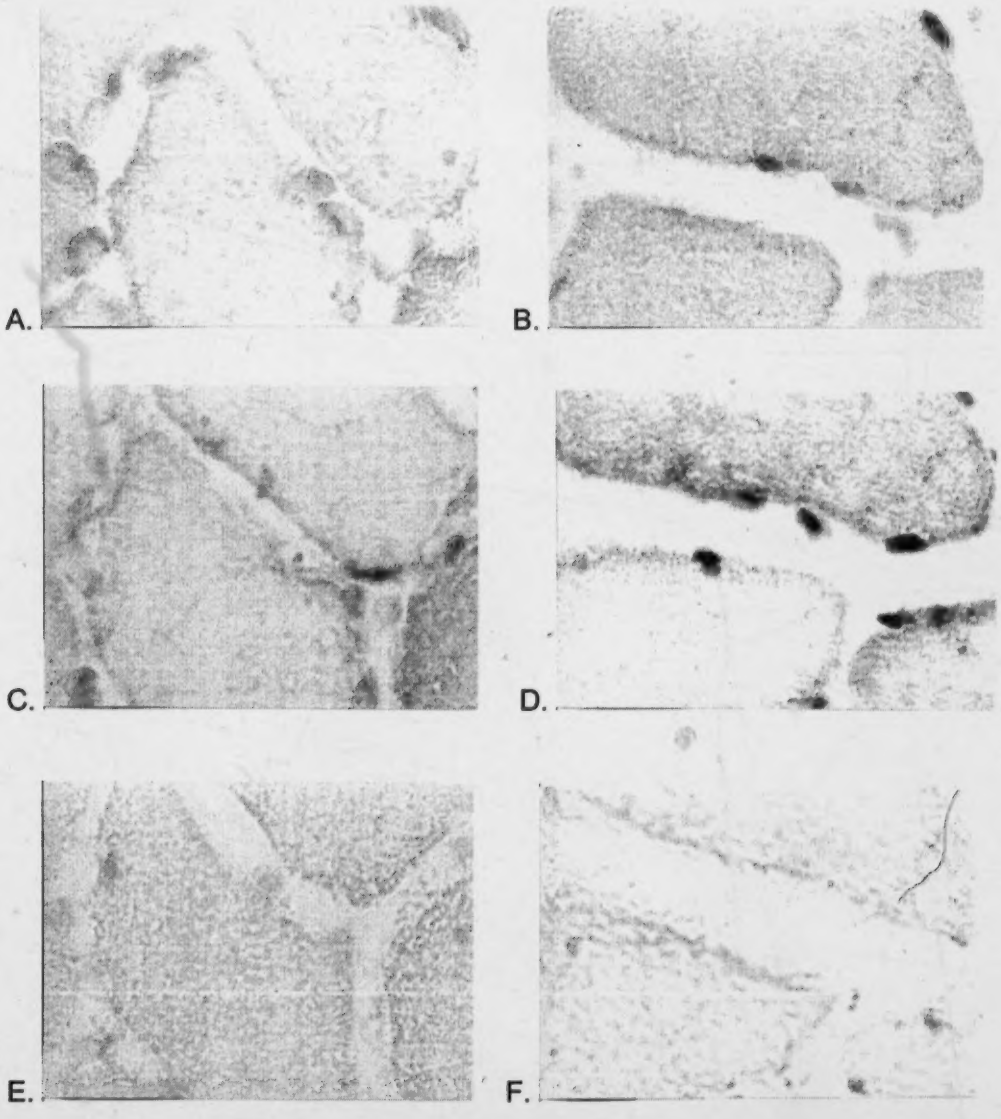

Figure IV-10: Immunohistochemical analysis of the isoform-specific nuclear distribution of AMPK in medial gastrocnemius (A,C,E) and soleus (B,D,F). Serial sections stained for: A&B: alpha1-AMPK, C&D: alpha-2-AMPK, and E&F: control (IgG).

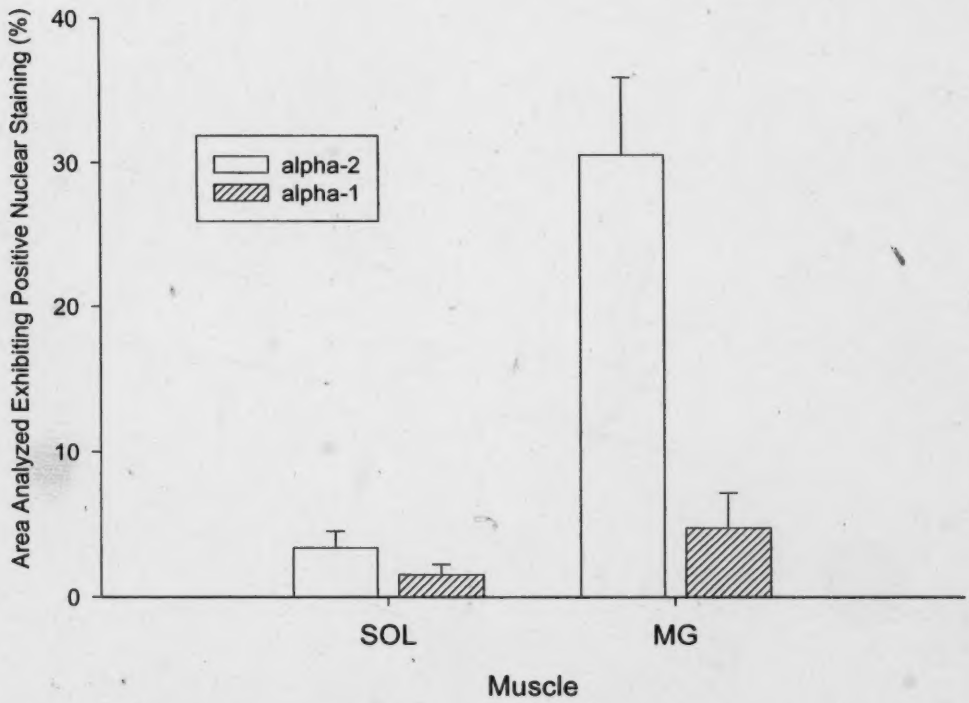

Figure IV-11: Isoform-specific expression of AMPK in the nuclei of medial gastrocnemius (MG) and soleus (SOL). Data analyzed by immunohistochemistry and light microscopy, expressed as percentage area analyzed exhibiting positive nuclear staining for alpha-1 or alpha-2 AMPK (mean area analyzed for each muscle: MG: 179.4mm^2, SOL: 74.3mm^2).

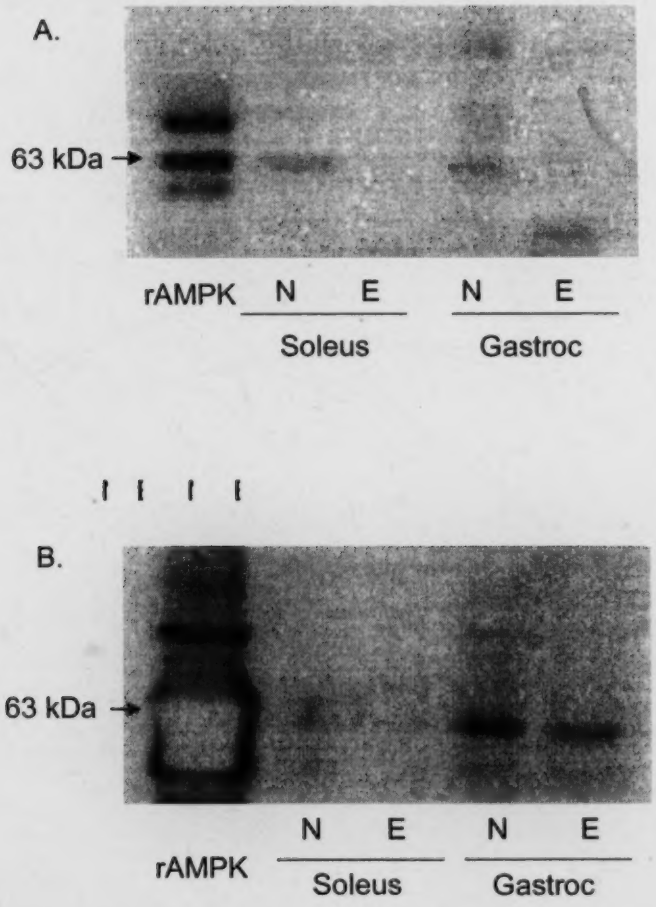

Figure IV-12: Representative Western Blot of nuclear (N) and extra-nuclear (E) fractions of medial gastrocnemius (Gastroc) and soleus (Soleus). Probed with **A.** anti-alpha-1 AMPK and **B.** anti-alpha-2 AMPK, recombinant alpha-1 and -2 proteins were used as internal controls respectively.

Table IV-2. Densitometric analysis of isoforms-specific AMPK content in nuclear and extra-nuclear fractions. Data are from western blots and are expressed as arbitrary densitometric units.

Fraction	α1	α2
Medial Gastrocnemius Nuclear	5.68×10^3	8.89×10^5
Medial Gastrocnemius Extra-Nuclear	2.48×10^2	4.58×10^4
Soleus Nuclear	6.21×10^3	5.98×10^3
Soleus Extra-Nuclear	2.59×10^2	1.77×10^2

Chapter V

Discussion

V.I Introduction

The purpose of the present study was to investigate fibre-type differences in the activity and distribution of AMPK in skeletal muscle. Available data suggest that AMPK may play a dual role in regulating both acute and chronic responses to perturbations in intracellular energy balance. Acutely, AMPK acts by increasing flux through ATP-producing pathways and attenuating ATP-consuming biosynthetic pathways. Chronically, AMPK appears to respond to reductions in cellular energetics by initiating adaptations that increase the metabolic efficiency of skeletal muscle fibres. This adaptive process is thought to ameliorate future disturbances to the intracellular energy balance (IEB). As skeletal muscle is a structurally and functionally complex tissue composed of a variety of types of fibres, an examination of the maximal activity and subcellular location of AMPK within the distinct slow and fast muscle fibre-types is fundamental to further understanding of the cellular role(s) of AMPK. The main finding of the present study was that both AMPK activity and expression were greatest in fibres expressing the fast-twitch MHC-IIb phenotype.

V.II Fibre-Typing of Rat Skeletal Muscles

The hypothesis was based upon the marked differences between the structural and metabolic characteristics of skeletal muscles fibres. The MHC expression of a fibre highly correlates with the mATPase activity (Staron & Pette, 1989), shortening velocity (Bottinelli et al. 1991; Galler et al. 1994) and tension cost (i.e. the relationship between ATPase activity and isometric tension) (Bottinelli et al. 1994). As such, it was first necessary to accurately determine the MHC based fibre-type of fast (white gastrocnemius), intermediate (red gastrocnemius), and slow-twitch (soleus) muscles. The expression of the MHC isoforms within these fibre-type populations was consistent with studies that have analysed rat skeletal muscles previously (Staron and Pette, 1993); separation of the mixed but primarily fast-twitch medial gastrocnemius into white and red portions allowed us to obtain distinct fast-twitch and intermediate-twitch muscles.

Electrophoretic analysis of the MHC expression confirmed the successful isolation of the gastrocnemius into fast and intermediate twitch portions. This separation was vitally important to facilitate the examination of AMPK in skeletal muscle possessing three distinct phenotypic profiles.

V.III AICAR as an Activator of AMPK

Although AICAR appears to be a non-specific activator of AMPK (Young et al., 1996), the ability to activate the kinase via elevated cellular ZMP levels in skeletal muscle without altering the concentration of nucleotides is

well documented (Holmes et al., 1999; Winder et al., 2000; Buhl et al., 2002). It has however, been noted that AICAR appears to be a more potent stimulus for increasing AMPK activity in fast-twitch fibres in comparison to mixed or slow fibres (Winder et al., 2000; Wojtaszewski et al., 2001). Based on the findings of the present study, the lower AMPK activity seen in the slow soleus and intermediate red gastrocnemius muscles (Figure IV-4) may be explained by the comparative absence of type IIB fibres in these muscles (Figures IV-1 and IV-3).

In addition, the tighter control that slow-twitch fibres hold over the IEB, and the ability to recover from acute perturbations furthers the reasoning as to why AICAR activates AMPK to a greater extent in white muscle. Following acute AICAR treatment, the decrease in the ATP-to-ZMP ratio would be more rapidly counteracted by combined ATP production from β-oxidation of fatty acids, glycolysis and glucose oxidation in more oxidative fibres.

Alternatively, Sabina et al. (1982) have suggested that slow fibres have a greater capacity to oxidize ZMP, which would account for the lower AMPK activity levels seen in red and mixed muscles despite a greater ZMP accumulation in comparison to fast-twitch muscles.

V.IV Total AMPK Activity is Highest in Fast-Twitch Fibres

An examination of both the isoform-specific and total activity of AMPK in different fibre-types was originally intended, however pilot work revealed a discrepancy between the combined activity of the immunoprecipitated α1 and

α2 fractions and the total activity. The immunoprecipitation procedure reduced the activity of AMPK by greater than 100-fold. Given this apparent limitation to the measurement of isoform-specific activity, the present study focussed only on investigating the total activity of AMPK in the distinct muscle fibre-types; quantitative determination of the α1 and α2 isoform specific contents were further examined by western blot.

To ensure maximal activation of AMPK, animals were acutely treated with AICAR (0.5 mg/g). While a dosage of 1.0 mg/g has been used previously to activate AMPK (Winder et al., 2001 and Holmes et al., 1999), recent studies (Buhl et al., 2002 and Bamford et al., unpublished data) have shown that 0.5 mg/g is sufficient to elevate ZMP levels and activate AMPK to the same extent. Following acute AICAR administration, the white gastrocnemius (which expressed ~79%IIb, 21%IId/x) displayed significantly greater maximal AMPK activity when compared to the red gastrocnemius (~18%IIb, 32%IId/x, 36%IIa, 14%I) or soleus (~22%IIa, 78%I). While other studies have investigated differences in AMPK activity between white and red muscle, the majority have employed immunoprecipitation and reported isoform-specific activity rather than total AMPK activity. However, the AICAR-induced activity of the α2 isoform of AMPK in rats has been shown to be fibre type-dependant as well, with the greatest activity in white gastrocnemius, lowest in soleus and the red gastrocnemius displaying an activity between the two (Wojtaszewski et al., 2002). Additionally, Jessen and colleagues (2003) have reported that

following treadmill exercise (20 $m \cdot min^{-1}$ at a 20% grade for 60 minutes) and AICAR stimulation, α2-AMPK activity was highest in muscle containing the highest proportion of IIB fibres in Wistar rats. These data are consistent with the results of the present study and suggest that muscles expressing greater amounts of α2-AMPK will have greater total activity.

Analysis of the AMPK activity in the present study in concert with the MHC expression patterns of the muscles supported this notion. While both the red and white gastrocnemius expressed type IID/X fibres, only the white contained the fastest twitch IIB fibres. Thus it appeared that the type IIB fibres were responsible for the majority of the AMPK activity in that muscle. The significant correlation found between the activity of AMPK and the percentage of IIB fibres ($r^2 = 0.92$, $p<0.05$) confirmed that the type IIB fibres are responsible for the majority of AMPK activity. This finding clearly supports the hypothesis that fast-twitch fibres would have the highest maximal activation of AMPK. That statement can now be made more specific, in that it appears that the greatest portion of AMPK activity within skeletal muscle occurs within the fastest IIB fibres.

V.V Isoform-Specific AMPK Content in Fast- and Slow-Twitch Muscle

While our activity data implied greater AMPK content in fast-twitch fibres, we also characterized the expression pattern of the two known isoforms of the AMPK catalytic subunits within fast, intermediate and slow-twitch fibres. Other studies have used Western blotting to analyse the distribution of AMPK in

skeletal muscles (Stapleton et al., 2000; Warden et al., 2001), only this study and the work of Ai and colleagues, (2002) which was published while the present study was in progress, have used both Western blotting and immunohistochemistry to characterize the fibre-type specific pattern of AMPK expression.

We first determined the fibre-type composition of the contralateral medial gastrocnemius and soleus muscles. Because it is possible to clearly discriminate type-I, -IIa, IId/x, and –IIb fibres with cross-sections of the whole gastrocnemius it was not necessary to separate the medial gastrocnemius into red and white fractions. Immunohistochemical fibre typing revealed results congruent with our electrophoretic results. The medial gastrocnemius was predominately composed of fast-twitch type-IIb and -IId/x fibres, but possessed all four fibre-types, whereas the soleus contained only slow-twitch type-IIa and -I fibres (Figure IV-1).

This cross-sectional analysis allowed the comparison of the distribution of AMPK on a fibre-by-fibre basis. While Ai and colleagues (2002) found no differences in staining between fibre-types, the present study found a fibre type-dependant-staining pattern (Figure IV-5). To our knowledge, this is the first time this has ever been reported. The reason for the differing results between the two studies can most likely be explained by the different fixation conditions used on the tissues. The aforementioned study fixed their tissues using methanol, however, initial experiments done in our laboratory revealed that no fixation was required to elicit optimal staining for AMPK, and that prior

fixation with methanol abolished staining. It is also of note that the Ai group opted to use antibodies raised according to the sequence reported by Woods et al. (1996), rather than the commercially available antibodies from Upstate Biotechnologies, which might also account for the difference in results due to differences in antibody performance.

The immunohistochemical data that was produced concurred with the activity data in that the distribution of AMPK was found to also be fibre-type dependent; with the fast-twitch fibres expressing greater AMPK content than the slow-twitch fibres. This supports the notion that the two known alpha isoforms of AMPK have different cellular roles. It must also be noted that immunohistochemical analysis appeared to show the α-2 staining to be of a slightly darker intensity in the type IIA and IID(X) fibres than in the type IIB fibres. This suggests that the α-2 content was also greater in those fibres, and if so, the α-2-AMPK present in the fast-twitch IIB fibres would have to have a much greater activation capacity to account for the marked difference seen in total activity between fast- and intermediate-twitch muscles. The reason for this discrepancy might be due to differences in antibody performance between the Western blot and immunohistochemical procedures. This possibility needs to be explored in future studies.

Being mindful of the limits of immunoprecipitation, several studies using this protocol have shown that the alpha-2 isoform, and not the alpha-1 to be activated following contractile activity in both rats (Vavvas et al., 1997) and

humans (Wojtaszewski et al., 2000; Fujii et al., 2000; Stephens et al., 2002; Yu et al., 2003). Following exercise in humans above 63% of VO_2max, the alpha-2 isoform of AMPK has consistently been activated from ~2.5- to 4-fold from basal levels, whereas the activity of the alpha-1 isoform remained at basal levels.

In a very recent study, isoform-specific AMPK activity was investigated in humans at three separate cycling intensities: low (40% VO_2max), medium (60% VO_2max) and high (80% VO_2max) (Chen et al., 2003). No significant increase in AMPK α1 or α2 activities from resting to low intensity exercise. Interestingly, AMPK α1 activity increased ~1.5-fold during exercise at the medium intensity but remained relatively unchanged during exercise at the high intensity. In contrast, AMPK α2 activity increased by ~5-fold during medium intensity and by approximately 8-fold during high intensity compared with resting levels.

These results, taken in concert with the data from the present study might well explain the activity of AMPK during exercise of increasing intensities from rest to 80% VO_2max simply by the recruitment pattern of the different fibre-types. It is well known that during exercise, muscle fibres are recruited according to the size principle, with smallest fibres (Type I) being recruited first, the largest fibres (Type II) recruited last, exercise at ~40% VO_2max would recruit only the smaller, slow-twitch type I fibres, and hence would not be sufficient to activate either alpha isoform of AMPK. As exercise

increases in intensity from 40-60% VO_2max, larger fibres would be recruited including the type IIA and IID/X fibres, which contain primarily α1 and only a small amount of α2 AMPK, eliciting increases in activity of both isoforms. Exercise at 80% VO_2max would recruit the largest, fast-twitch IIB fibres, resulting in a further activation of the α2 isoform. The activity of α1 AMPK would plateau due to the fact that all the fibres containing the α1 isoform would have already been recruited.

The Western blot data also revealed a fibre-type specific distribution of AMPK and supported our immunohistochemical findings. These data seem to suggest that muscles expressing a large complement of MHC IIB have the highest α2-isoform content, while expression of the alpha-1 isoform is greatest in fibres that have a high MHC-I content.

Interestingly, a double banding pattern was found on the both of our blots, with one band being located at the 63 kDa molecular weight, and one just above it. While the bands are slightly further apart on the alpha-2 blots, the banding pattern is the same on both. This double banding phenomenon has been mentioned previously in a study by Salt and colleagues (1998) studying AMPK in hepatocytes, and is most likely due to the phosphorylation of AMPK. The phosphorylated AMPK would migrate slightly further down the gel during electrophoresis due to the addition of the phosphate. This occurrence might explain why the white gastrocnemius has the strongest upper band in comparison to either the red gastrocnemius or the soleus.

V.VI Isoform-Specific Subcellular Localization of AMPK in Fast- and Slow-Twitch Muscle

In the present study, immunohistochemical analysis revealed positive staining for both α-isoforms in the soleus and gastrocnemius. While staining for both the α1- and α2-isoforms was very limited in the soleus, there was a ~6-fold greater occurrence of positively stained nuclei for α2 over α1 in the gastrocnemius (Figure 4-10). Western blotting confirmed this, showing the same ~6-fold greater expression of α2 in the nuclear fraction when compared to the extra-nuclear fraction in the medial gastrocnemius (Table 4-2). These data are similar to results found by others who have reported a nuclear localization of the α2-containing complex of AMPK in both hepatic tissue (Salt et al., 1998) and skeletal muscle (Ai et al., 2002).

The presence of the α2-isoform in the nuclei of the fast-twitch fibres would suggest that it might play a role in altering phenotypic expression. AMPK has previously been linked to affecting the phenotypic transformation of muscle by increasing the expression of both glycolytic (Suwa et al., 2003) and oxidative enzymes (Suwa et al., 2003; Winder et al., 2000), increasing mitochondrial biogenesis (Zong et al., 2002), GLUT4 (Maclean et al., 2002), and PGC-1 (Peroxisome proliferator-activated receptor-γ coactivator 1α), a transcriptional co-activator that interacts with several nuclear transcriptional factors, itself promoting GLUT-4 expression (Michael et al., 2001),

mitochondrial biogenesis and fibre-type transformation (Lin et al., 2002) in skeletal muscle cells.

In the present study, the α2-isoform was not found to be located in the nuclei of the slow-twitch soleus to the same extent as the medial gastrocnemius. This seems to support the hypothesis that slow-twitch fibres have less need for AMPK-mediated phenotypic alterations due to a lesser capacity for fast-to-slow transformation than do fast-twitch fibres. That is, fibres which are more metabolically efficient, fatigue resistant and located further toward the slow-twitch end of the fibre-type continuum would require less adaptation, and therefore less nuclear AMPK than fast twitch fibres.

Interestingly, a very recent study by McGee and colleagues (2003) found that following 60 min of cycling at 72% of VO_2peak nuclear AMPK α2 content was increased 1.9-fold while there was no change in whole-cell AMPK α2 content or AMPK α2 mRNA abundance. This observation supports the hypothesis that the effects of exercise on skeletal muscle gene and protein expression might be mediated by the nuclear translocation of AMPK.

V.VII Conclusions

Success within the natural world requires the ability to respond to acute stress, but to also adapt to repeated stressors. Organisms with exceptional adaptive responses persevere, while those lacking this ability perish when environmental situations are altered. Therefore, it is logical that a complex

system governs the fundamental process of maintaining cellular energetics. Research continues to support the notion that the highly conserved AMPK cascade plays a crucial role in regulating this process, but the complexity of the pathways affected by this multi-substrate kinase remains unclear.

Nevertheless, the findings of the present study add to the body of knowledge on AMPK, by suggesting that due to the inability to maintain their cellular energy balances, fast-twitch fibres appear to require greater AMPK activity to counteract acute perturbations to cellular energetics, including bouts of exercise. These same fast-twitch fibres also display a greater amount of nuclear AMPK; furthering the notion that AMPK plays an essential role in signalling the phenotypic fast-to-slow transformation that occurs in skeletal muscle following chronic stimulation. While the present study is to the author's knowledge, the first to show the fibre-type dependent distribution of AMPK on a cell-by-cell basis, the apparently specific roles of the two α-containing isoforms of AMPK remain unclear.

Chapter VI

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Education

University of Alberta
Master of Science (MSc) in Exercise Physiology - 2004
Faculty of Physical Education

University of Alberta
Bachelor of Physical Education (BPE) with distinction - 2000
Sport Performance specialization
Faculty of Physical Education

Employment

Body by Bennett Fitness Studio – Edmonton, Alberta
Responsibilities included all aspects of design, implementation, and management of fitness training programs including mind-body wellness to various populations, also designed and implemented a program of corporate fitness and wellness educational seminars.

Sport Performance Unit – University of Alberta
Exercise testing including maximal aerobic power, maximal anaerobic power, maximal strength, muscular endurance, and body composition, as well as exercise prescription for various populations.

The Presence: **Elite Sport Performance** – Edmonton, Alberta
Responsible for all aspects of design and implementation of elite athlete training programs.

Refereed Publications and Juried Exhibitions

Dissertation
Ryan J. Saranchuk. Activity and Isoform Specific Distribution of AMP-Activated Protein Kinase in Rat Skeletal Muscle of Different Fibre-Types, Master of Science Thesis, University of Alberta, 2004. I examined one possible mechanism responsible for the adaptive transformation that occurs in skeletal muscle following chronic stimulation or endurance training, resulting in a more energy-efficient, oxidative fibre.

Published Abstracts: Refereed Journals

R. Saranchuk, J. Altarejos, M. Gallo, G.D. Lopaschuk and C.T. Putman. Activity of AMP--Activated Protein Kinase in Skeletal Muscles Composed of Different Fibre-Types. *Canadian Physiology Society (2001).*

R. Saranchuk, M. Kiricsi, J. Altarejos, I. MacLean, G.D. Lopaschuk and C.T. Putman. AMP-Activated Protein Kinase Activity is Greatest in Muscle Composed of Fast-Twitch Type-IIB Fibres.
Federation of American Societies for Experimental Biology (FASEB)(2002).

K. Martinuk, **R. Saranchuk**, M. Kiricsi and C.T. Putman. Isoform Specific Fibre-Type Distribution and Subcellular Location of AMP-Activated Protein Kinase in Rat Skeletal Muscles. *International Biochemistry of Exercise (2002).*

Conference Presentations

Oral Presentation: Activity of AMP-Activated Protein Kinase in Skeletal Muscles Composed of Different Fibre-Types.
Canadian Physiology Society Meeting, Silver Star B.C. (2001).

Poster Presentation: AMP-Activated Protein Kinase Activity is Greatest in Muscle Composed of Fast-Twitch Type-IIB Fibres.
Federation of American Societies for Experimental Biology (FASEB), New Orleans, Louisiana (2002).

Poster Presentation: Isoform Specific Fibre-Type Distribution and Subcellular Location of AMP-Activated Protein Kinase in Rat Skeletal Muscles.
International Biochemistry of Exercise, Maastricht, Holland (2002).

Professional/Research Experience

Research Assistant - Faculty of Physical Education, University of Alberta, Edmonton, Alberta. **The effects of hyperoxia on maximal and submaximal exercise performance**
Responsible for administering maximal and submaximal assessments to individuals wearing self-contained breathing apparatus (SCBA) and breathing a hyperoxic (40% O_2) gas mixture. All tests were performed with metabolic gas analysis. Supervised by Dr. Stewart Petersen (780-492-1026).

Research Assistant - Faculty of Physical Education, University of Alberta, Edmonton, Alberta. **Validation of the 20 meter shuttle run test**
Responsible for administering 20 meter Leger Shuttle run to both male and female participants. Supervised by Dr. Stewart Petersen (780-492-1026).

Research Assistant - Faculty of Physical Education, University of Alberta, Edmonton, Alberta. **Effect of breathing through an SCBA versus a Hans Rudolph valve during maximal exercise**
Responsible for administering maximal assessments to individuals wearing either an SCBA or a Hans Rudolph valve. Supervised by Dr. Stewart Petersen (780-492-1026).

Teaching Experience

University of Alberta
Applied Resistance Training
Terms: Summer 2001, 2002
Lecturer: Responsible for instructing senior undergraduate students in a course designed to further understanding of the theory and practice of resistance training. Specifically, optimizing resistance training to elicit specific adaptations to various stimuli.

Advanced Conditioning Methodology
Terms: Winter 2003
Invited Lecturer: " Principles of Effective Aerobic Training for Endurance Athletes"..

Advanced Conditioning Methodology
Terms: Winter 2003
Invited Lecturer: " Principles of Effective Resistance Training for Different Types of Athletes".

Anatomy
Term: Winter 2000, Fall 2001
Laboratory Instructor: Responsible for instructing undergraduate students in a course designed as an introduction to basic human anatomy.

Exercise Physiology
Term: Winter 2001, Fall 2002
Laboratory Instructor: Responsible for instructing undergraduate students in a course designed as an introduction to the basics of exercise physiology.

Achievements and Awards

Represented Alberta in Rugby at the Canada Summer Games	1993
Selected to Under 19 Rugby Canada Team	1994
Member of the U of A Golden Bears Hockey Team	1997-1998
National Coaching Certification Program level II - technical	2000
National Coaching Certification Program level I - practical (rugby)	1993
Jimmy Condon Scholarship for athletic and academic achievement	1997-1999
CIAU Academic All-Canadian U of A Hockey	1997-1999
Louise McKinney Scholarship for academic excellence	1999

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